Functional aspects of seasonal variation in preen wax composition of sandpipers (Scolopacidae)

The research in this thesis was carried out at the
Royal Netherlands Institute for Sea Research (NIOZ),
PO Box 59, 1790 AB Den Burg, The Netherlands

with financial support from the
Netherlands Organisation for Scientific Research (NWO-ALW)

Printing of this thesis was made possible with financial support
from the J.E. Jurriaanse Foundation

COLOFON

Layout:	Dick Visser
Cover layout:	Jeroen Reneerkens and Welmoed Ekster
Drawings:	Welmoed Ekster
Photographs:	Jan van de Kam (pages 13, 16, 92, 105) Jolanda van Iperen (page 109) Jeroen Reneerkens (pages 117, 152)
Print:	Van Denderen bv, Groningen

ISBN:	90-367-2918-1
ISBN digitaal:	90-367-2919-x

RIJKSUNIVERSITEIT GRONINGEN

Functional aspects of seasonal variation in preen wax composition of sandpipers (Scolopacidae)

PROEFSCHRIFT

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. F. Zwarts,
in het openbaar te verdedigen op
vrijdag 12 januari 2007
om 16.15 uur

door

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geboren op 26 april 1975
te Sittard

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Summary

The uropygial gland, also called preen gland, is commonly found in birds. Waxes secreted from the gland are spread over the plumage with birds' bill during plumage maintenance. In captive red knots *Calidris canutus*, plumage maintenance took on average 7.2% of the time, but only 2.9% of all preening bouts involved the application of preen wax (box A). The biological function of the preen waxes have been debated for many centuries and their chemical composition studied rather extensively, but both fields of research were never combined (reviewed in chapter 1). The scientific debate got a new impulse with the discovery that in red knots, sandpipers that winter in tropical or temperate coastal intertidal areas and that breed in the High Arctic, the chemical composition of their preen gland secretions changed prior to departure on a long-distance flight to the breeding grounds in spring. Within a few days, at the level of a population, the secretions consisting of monoesters only were replaced with a preen wax mixture of pure diesters. The selection pressures that have led to the seasonally changing preen wax composition in sandpipers are the focus of this thesis. To examine the putative selection pressures, comparative and experimental, biological and chemical studies were carried out both in the field and in the laboratory.

At least 18 species of sandpipers other than red knots also showed preen wax composition seasonally changing from mono- to diester preen waxes. Diester secretion extended beyond the period of courtship and mate choice and continued throughout incubation. At hatch, the secretions reverted to the usual monoester waxes (chapter 2). All investigated sandpipers secreted species-specific mixtures of monoesters and diesters. The diesters of most sandpipers can be divided in two groups; diesters that are based on 1,2-diols and diesters based on ß-hydroxy fatty acids, both of which may occur in the secretion of a single individual, but in different percentages per species (box B).

This drastic shift in preen wax composition naturally leads us to wonder about its biological function. In chapter 3, the comparative study as described in chapter 2 was extended by studying seven sandpiper species with different incubation patterns, including species where both sexes incubate, only males incubate, or where only females incubate. During the breeding period, diester preen

wax was secreted almost exclusively by the incubating sex in species with uniparental incubation, and by both sexes in species with biparental incubation. These findings clearly suggest that diester preen waxes have a function that is directly related to the act of incubation. Some male curlew sandpipers and buff-breasted sandpipers – species with female-only incubation – nevertheless secreted diester preen waxes during the breeding period. These exceptions to the rule that diester preen waxes are only secreted by incubating individuals can be explained if these diester waxes are a remnant from an evolutionary past when both sexes in these two species incubated. An alternative explanation could be that males need to be olfactory cryptic (a function enhanced by diester preen wax which is less volatile than monoester wax; see below) because they are involved in the making of nest scrapes that will later be used by the females.

The secretion of diester preen waxes seems particularly functional during incubation, but probably entail certain net costs in other periods. A close match between the secretion of diester waxes and incubation would avoid redundant costs, but requires a flexible temporal organisation of preen wax shifts. The flexibility of seasonal cycles in preen wax composition, relative to better understood seasonal cycles in body mass and moult, were investigated in chapter 4. Two experimental perturbations were used to get insight in the (constraints in) flexibility of preen wax changes: (1) giving birds restricted access to food and (2) monitoring them long-term under a constant photoperiodic regime. Red knots subjected to a constant photoperiod continued to show seasonal changes in preen wax composition. These rhythms became free-running and diester secretion was temporally correlated with changes in body mass, suggesting that preen wax shifts, like seasonal changes in body mass and moult, are under endogenous control. However, diester preen wax secretion was more poorly expressed in the experimental treatments. That complete changes from mono- to diester preen waxes in captive red knots took on average 30 days, together with the fact that these changes are endogenously controlled, showed that preen wax shifts are similarly time-constrained as seasonal changes in body mass and moult.

It has been hypothesised that the shift to diester preen waxes by red knots in spring is a sexually selected trait that enhances birds' plumage appearance, signaling individual condition during the period of mate choice. This hypothesis assumes that a compositionally different preen wax coat alters the light reflectance (colour) of the plumage. This assumption was tested in chapter 5 by the use of spectrophotometry of plumages of six red knots before and after they had undergone a complete shift from monoesters to diesters in spring. In addition, preen wax was (partially) removed from the plumages after which its reflectance was measured again, to make sure that possible changes in plumage reflectance are attributable to the shifts in preen wax composition only. Light absorbance by the

pure monoester and diester waxes was also measured. Although diesters absorbed more light, especially in the ultraviolet light spectrum, a shift in plumage reflectance was not observed with a change in preen wax composition. Because a small change in hue and chroma of the plumage with preen wax change was also observed in the plumages after preen wax removal, it can not be attributed to the (shift in) preen wax. I conclude that the layer of preen wax on the plumage is too thin to bring about a change in colouration.

In contrast to the expectations, an observed change in plumage colouration of female lapwings, appeared not to be caused by changes in preen wax composition. Lapwings (both sexes), dotterels and Kentish plovers, secrete monoester preen wax year-round and are thus unexplained exceptions to the rule of seasonal changes in preen wax composition in shorebirds (box C).

In chapter 6 we report on the effect of monoester and diester preen wax on the growth of a feather-degrading bacteria *Bacillus licheniformis*, shown to occur in plumages of red knots also. As growing conditions for these bacteria are presumably better in the nest cups of incubating sandpipers that are relatively warm and humid, it was hypothesised that diesters would offer a better protection against these bacteria. A difference in break-down of breast feathers of red knots, applied with mono- or diesters by the birds themselves was, however, not observed. The removal of preen waxes (mono- or diesters) from feathers resulted in faster degradation of the feathers, confirming earlier studies that preen wax inhibits growth of feather-degrading bacteria. In a field experiment we did not find evidence for the idea that (diester) preen waxes protect wing feathers against (physical) abrasion (box D).

A trained sniffer dog was used in an experimental set-up to test whether diester preen waxes are more difficult to detect by smell than monoester preen waxes (chapter 7). The olfactory-searching dog indeed had greater difficulty detecting mixtures of the less volatile diesters than mixtures of monoesters. This is consistent with the hypothesis that diester preen waxes reduce birds' smell and thereby reduce the risk of predation of their clutches.

In chapter 8 the main findings of the research described in this thesis and suggestions for future research on (seasonal) variation in preen wax composition are discussed in a broader context. In brief, I conclude that more can be learnt about variation in preen wax by looking at a broad scale for patterns in preen wax composition with species' habitats, breeding system and phylogeny. Also, knowledge of the physical aspects of preen wax composition and of the (causes of) turn-over rates of preen wax once applied onto the plumage, is still limited. A combination of chemistry, physics and biology will increase our understanding of the large variation in preen wax composition between species and within individuals.

CHAPTER 1

Functional aspects of variation in preen wax composition

Jeroen Reneerkens

It is the possession of feathers that makes an organism a bird (Sumida & Brochu 2000). More than twenty functions of feathers have been suggested (Stettenheim 1976, 2000). Feathers enable birds to fly (Homburger & da Silva 2000) and thus to have large home ranges to forage in, and the capacity to seasonally migrate and to escape from life-threatening predators. Feathers also provide an essential thermal buffer between the animal and its environment (Wolf & Walsberg 2000), and by their colour patterns and form, feathers are important for communication with conspecifics (Burtt 1979).

Feathers function better when they resist excessive wear and tear. Nevertheless, they still need replacement at regular intervals; a process called moult. Feather wear occurs by exposure to ultraviolet light, contact with hard objects such as vegetation and airborne particles and by ectoparasites such as feather lice and some feather mites that chew holes in feathers (Proctor & Owens 2000; but see Blanco *et al.* 2001 who argue that most feather mites may not be parasitic) and feather-degrading bacteria that break down feather keratin (Burtt & Ichida 1999).

Because of the important functions of feathers and the many factors that may cause wear, it is perhaps not surprising that birds spend a significant time of the day maintaining their plumage. Plumage maintenance involves nibbling the feathers by the bill, probably to remove ectoparasites and alien particles from the feathers and also to arrange and reattach feather barbs that hook into each other for the feathers to remain an optimal (thermal) barrier between bird and its environment (Wolf & Walsberg 2000).

An important aspect of daily maintenance is the lubrication of feathers with fatty secretions. Next to lipid secretions from the skin (Menon & Menon 2000), lipids from a specialised organ, the uropygial gland, also called the preen gland (Elder 1954, Jacob 1976, Jacob & Ziswiler 1982), are smeared onto the plumage. Secretions originating from the skin and the preen gland consist of different chemical compounds and their relative amount on feathers differs among bird species (Stettenheim 2000).

Almost all bird species posses a preen gland. All ratids (Struthionidae, Rheidae, Casuaridae, Dromaidae) lack a preen gland, and preen glands are also absent in a few species of Columbidae and Psittacidae (Johnston 1988) and members of the orders Galliformes, Gruiformes, Caprimulgiformes and Apodiformes (Elder 1954). The preen gland is a small organ located at the base of the tail that consists of two (partially) subdermal lobes which are composed of numerous holocrine secretory alveoli that open in a central cavity (Lucas & Stettenheim 1972, Jacob & Ziswiler 1982, Stettenheim 2000) from which the wax secretion is transported to the surface of the gland via ducts that open at the top of a pappilla, a nipple-like tip of the gland that in several species is provided

Figure 1.1 A preening red knot touches its preen gland with swift movements of the bill. Its lower back feathers have been raised to make the gland accesible to the bill.

with a small feather tuft that presumably aids in the distribution of the waxes onto the bill tip during preening (Jacob & Ziswiler 1982).

During preening activities, often (or only: van Rhijn 1977a) soon after bathing, the tail is spread and turned towards the head and the lower back feathers near the gland are raised so that the gland becomes visible and accessible for the bird's bill (fig. 1.1). The bird then collects the secretions from the gland by touching it with rapid, repetitive movements onto the bill after which preening activities follow and the waxes are distributed over different parts of the plumage. As the feathers on the head can not be reached by the bill, the head is usually provided with wax by rubbing it on the shoulders after which these have been supplied with fresh wax secretions. This may transfer the preen wax onto the head. Subsequently, the preen wax may be further distributed evenly over the head feathers by scratching with the birds' toes. Sometimes the head is rubbed directly against the preen gland. Primary wing feathers are lubricated with preen wax by moving the primaries between the bill mandibles from the base to the tip of the feather in rapid movements after wax from the gland has been obtained on the bill tip. Van Rhijn (1977b) provides a more detailed description of preening

activities by a herring gull *Larus argentatus*. One should realise that, although preening activities take considerable time in the daily time budget of birds (5-30%; references in Haribal *et al.* 2005), it only partially involves application of preen waxes onto the plumage. Preening bouts of barn swallows *Hirundo rustica* involved contact with the preen gland in just 3.1% of the observed cases (Møller 1991), a similar percentage as found in captive red knots *Calidris canutus* (box A). Nevertheless, plumages normally contain significant amounts of lipids that presumably originate from the preen gland. Hou (1928) showed that small bundles of feathers lost about 5% in weight after fat extraction, suggesting that birds' feathers contain significant amounts of fatty substances, which are likely to originate from the preen gland. More recently, Bollinger & Varga (1961) found that feathers contained *ca.* 2% of lipid material extractable with ether or chloroform.

Context of this thesis

Preen gland secretions have been subject of studies by biologists and chemists. Biologists were interested in their biological functions and studied preen waxes without paying much attention to their chemistry. Chemists, on the other hand, studied the chemical composition of the waxes, but only occasionally wondered about their functions.

Functions of preen waxes are tightly linked with their chemical composition that determines physical (Patel *et al.* 2001, Kulkarni & Sawant 2002) and thereby biological aspects of the secretions. Variation in chemical composition results in different functions of preen wax. Vice versa, the physical aspects and biological functions are subject to natural selection which will result in variation in chemical composition. For example, melting temperatures and viscosity are determined by the wax composition (Kulkarni & Sawant 2002) and affects the viscosity and thereby the 'smearability' of the secretions onto the plumage. If, for simplicity reasons, all other possible aspects of preen wax are ignored, it is to be expected that natural selection will result in a preen wax with a for birds optimal viscosity. The hydrophobic properties are, amongst several other chemical aspects, determined by the degree of branching of the fatty acids of which preen waxes are composed. According to Sweeney *et al.* (2004) branched fatty acids are less water-soluble and might thus aid better in waterproofing the plumage.

Below, the main findings of several centuries of biological and chemical preen wax research will be reviewed seperately. Then, the research subject of this thesis will be described; a biological study to the function of intra-specific seasonal variation in preen wax composition.

Functions of preen gland secretions: the biologists' approach

Waterproofing

Despite discussions about the function of preen glands in birds since at least the 17th century (Elder 1954), there still is much speculation about, and little unequivocal experimental evidence for, the functions of uropygial gland secretions (e.g. Jacob 1976, 1978, Elder 1954 and references therein). As the wax is smeared onto the plumage, the most often suggested functions of the wax involve plumage maintenance. Waxes are hydrophobic and are likely to contribute to the waterproofing of birds (e.g. Elder 1954, Jacob & Ziswiler 1982), in addition to the avian epidermal lipids (Menon & Menon 2000). The feather keratin is about as water repellent as preen wax and feathers are also water repellent by their microstructure (Rijke 1970, Elowson 1984). In several studies preen glands were surgically removed from birds, usually at an age of a few days, to look for the effects on their adult plumages especially in relation to water retention in the feathers. Remarkably, most of these studies reported no visual effects on the plumage and in most of the cases the birds survived without preen glands, at least for some months up to several years (Elder 1954, Jacob 1976, Chen *et al.* 2003). It was, however, noted that the feathers of glandless birds appeared exceptionally brittle and less glossy in some cases, an effect that –surprisingly- disappeared after the first moult following preen gland removal. Elder (1954) reports of an experiment in which the water retained in the plumage of ducks after submersion in water was compared between ducks of which the preen glands had been removed and control birds. After the birds were allowed to dry for fifteen minutes, the plumages of the glandless birds appeared to contain twice as much water as those of the control birds. These results could, however, not be confirmed in a similar experiment in which it was shown that the amount of water repellence of plumages was strongly related to the time spent preening, irrespective of the application of preen wax onto the feathers (Fabricius 1959). This suggests that feather arrangements and closing of the barbules of feathers play a more important role in water repellence than the application of waxes onto the feathers. Rutschke (1960) showed that experimental removal of lipid components from the belly feathers of ducklings and adult ducks did not affect the buoyancy, but it has been questioned whether all of the fat components were removed from the feathers (van Rhijn 1977a). In an experiment, van Rhijn (1977a) compared the water absorption of several different feathers of a herring gull before and after he removed preen wax with benzene and ethanol, but found significant higher water absorption after preen wax removal in only three of 20 pairwise comparisons. An estimate of the amount of fatty substances in feathers, by use of Sudan III stain, showed no relation with the degree of waterproofing of the feathers. Furthermore,

Plumage of birds, repels water by its microstructure. The contribution of preen waxes to this waterproofing effect is still unclear.

a negative correlation between feather weight and water absorption was found, and it was suggested that feather size and microstructure probably play a greater role in waterproofing than the occurrence of preen wax (van Rhijn 1977a).

Perhaps, the unconfirmed general belief that preen wax is especially important for water repellence of feathers stems from the fact that preen glands are relatively large in many aquatic bird species, such as the American dipper *Cinclus mexicanus*, a waterbird that lives in small streams in which it regularly plunges to catch prey, that possesses a preen gland that comprises 0.71% of its body mass (Montalti & Salibián 2000). Relative sizes of preen glands vary between 0.014 and 0.556% in 1164 adult birds (126 species, 49 families). Jacob & Ziswiler (1982) already showed that relative preen gland weights constituted between 0.05% and 1.14%. The pattern of waterbirds having relatively large preen glands was already noticed in 1860 (Elder 1954), but a clear relationship between the relative preen gland weight of bird species and the degree of their use of aquatic habitats could not be confirmed (Montalti & Salibián 2000).

Flexibility of feathers

It has also been suggested that preen gland secretions protect feathers by delaying abrasion and/or by keeping feathers flexible (e.g. Jacob 1976, Jacob & Ziswiler 1982). As far as I am aware, efforts to investigate the effects of preen waxes on abrasion resistance of feathers have never been undertaken. Rutschke (1960) showed that preen waxes quickly penetrate into the medulla cells of the shaft and barbs of feathers and concluded that the wax may play a role in keeping feathers' elasticity. Preen wax may then indirectly contribute to waterproofing

(and thermal insulation) of the plumage as this depends on tiny air bubbles that are kept within the small meshwork of feather barbs (Rijke 1970, Rijke *et al.* 1989). It is also imaginable that feather barbs and shafts break less easily when they are more flexible.

Skin and bill condition
Ducks of which preen glands were removed when several days old had, next to a less shiny and dryer looking plumage, a dry, peeled surface of bills, legs and feet. The effect on the plumage disappeared after eclipse moult, but the effects on the bare unfeathered parts remained, suggesting a function of preen waxes on skin and bill too (Elder 1954).

Anti-parasitic effects
Feathers of birds often host many different ectoparasites such as ticks, lice and mites that are usually detrimental to their host, especially when they occur in high densities (Clayton 1990) as they may damage the plumage, suck blood from their host or act as disease vectors (Scullion 1989, Møller *et al.* 1999). Removal of ectoparasites from the plumage with the bill is difficult (Blanco *et al.* 1997, 2001). Hence, birds use chemicals that may help against ectoparasites (Hart 1997). Examples are the rubbing of secretions of ants' metapleural glands into feathers, a behaviour known as "anting" (Ehrlich *et al.* 1986, Clayton & Vernon 1993, Revis & Waller 2004) and the use of green plant material in nests (Clark & Mason 1985, 1988, Gwinner *et al.* 2000, but see Brouwer & Komdeur 2004 who did not find evidence for anti-parasitic effects of the use of green plant material in starling *Sturnus vulgaris* nests). In addition to the use of such exogenous resources, there are examples of endogenously produced chemicals. Specialised avian epidermal lipids (Menon & Menon 2000) have been shown to be toxic to ectoparasites (Douglas *et al.* 2001, 2004, 2005a,b, Dumbacher & Pruett-Jones 1996, Dumbacher 1999, Dumbacher *et al.* 1992).

Not surprisingly, preen waxes are supposed to have anti-parasitic effects too (e.g. Jacob *et al.* 1997). Moyer *et al.* (2003) showed that feather-feeding lice (Phthiraptera: Ischnocera) died more rapidly on feathers of rock doves *Columba livia* with preen waxes than on feathers without, suggesting that waxes aid in combating lice. However, as the removal of the preen gland from captive doves had no significant effect on louse loads over the course of a four-month experiment, it is unclear whether the preen wax also negatively effects the survival of louse under natural conditions. Microbes, such as bacteria (Böckle *et al.* 1995, Burtt & Ichida 1999, Sangali & Brandelli 2000) and fungi (Santos *et al.* 1996) on the feathers of birds are able to degrade feathers in vivo, but their degrading effects on live birds have been questioned (Cristol *et al.* 2005). Preen wax of house

finches *Carpodacus mexicanus* inhibited growth of five bacterial isolates that degraded feathers either strongly, weakly or not at all. The wax had no effect on seven other isolates and enhanced growth of one weak feather-degrading bacterium (*Micrococcus nishinomyaensis*, although no data exist of this bacterial strain about effects on birds; Shawkey *et al.* 2003). Similar results, of suppression of some and enhancement of growth of other skin surface bacteria, as well as fungi, were found in chicken (Bandyopadhyay & Bhattacharyya 1996, 1999). Although, the effects of preen wax on microbial communities on feathers and skin are not unambiguous and rather complex, they suggest that preen wax might act as a defense mechanism against detrimental bacteria in birds (Jacob & Ziswiler 1982, Shawkey *et al.* 2003). Anti-microbial effects of preen wax were also proposed by Jacob *et al.* (1997), but this conclusion resulted from experiments based on hydrolysis products of preen wax (alcohols and fatty acids, see 'Chemical composition of preen wax; the chemists' approach') which have very different chemical and biological properties than the intact waxes that are smeared onto the plumage. Not only may preen wax have effects on ectoparasites, it may also affect harmless or beneficial organisms living in birds' plumage, such as feather mites that are presumed to feed on the feather wax and may even remove 'old' wax (Dubinin 1951, Blanco *et al.* 2001). It is, however, unclear what Blanco *et al.* (2001) counted as 'old wax' and what it implicates.

Besides effects on organisms living in the plumage of birds, preen waxes might also attract or repel flying insects. For instance, preen wax of American crow *Corvus brachyrhynchus* attracts Theobald mosquitoes (Diptera: Culicidae), that feed primarily on birds and are known to be the major vector of West Nile virus in North America (Russel & Hunter 2005). On the other hand, odorous secretions of crested auklets *Aethia cristatella*, presumably originating from a specialised gland-like structure associated with the integument (Douglas *et al.* 2001), has been shown to repel mosquitoes (Douglas *et al.* 2005b).

Smell

Smell is the reception of information from a source that releases volatile components. The volatility of these components determines the distance from the source over which the information can be received. The composition of the volatile compounds further determines how the information is experienced (e.g. malodorous, sweet or otherwise 'smelling good'), and whether the source attracts or repels recipients of the smell. As preen wax attracts mosquitoes (Russel & Hunter 2005), it is clear that the wax has a certain smell that can be detected by animals. Preen wax of most bird species, however, lacks a smell obvious to humans. This obviously does not imply that preen wax may not play a role in olfactory communication between birds, which have been shown to have a functional

sense of smell (Roper *et al.* 1999). It has been suggested that the preen wax products of female mallards *Anas plathyrynchos* may be involved in pheromonal communication between female and chicks (Jacob *et al.* 1979, Balthazart & Schoffeniels 1979). It should be noted though that preen waxes are not very volatile and thus not the most obvious chemical compounds to play a role in olfactory communication.

A well-known example of a penetrating preen wax secretion is that of hoopoes *Upupa epops*. The composition of the secretion becomes darker and more malodorous in females and nestlings during the the nesting phase, creating a bad smell in the nests. The preen wax of nesting hoopoes repels feather-degrading bacteria (Martin-Platero *et al.* 2006) and presumably helps to repel vertebrate predators (Elder 1954). Red-billed woodhoopoes *Phoeniculus purpureus* produce a penetrating, black preen wax resulting from a coccoid bacterium that lives in the preen gland in symbiosis with the birds and breaks down the preen wax secretions into 17 preen wax compounds, thereby changing colour, viscosity, volatility and smell of the secretion (Law-Brown 2001, Law-Brown & Meyers 2003). Seven of the 17 identified compounds showed inhibitory action against 13 species of pathogenic bacteria and one parasitic bacterium (Law-Brown 2001).

A more recent study identified at least 52 compounds in the preen gland secretion of red-billed woodhoopoes, also called green woodhoopoe (Burger *et al.* 2004). In addition to anti-bacterial effects, the malodorous compounds have also been shown to repel vertebrate predators of eggs (Law-Brown 2001). Strong odorous secretions of crested auklets, presumably originating from specialised epidermal cells (Douglas *et al.* 2001), have been described to repel different tick species (Douglas *et al.* 2004) and repel mosquitoes (Douglas *et al.* 2005b). Alternatively, the citrus-like smell of crested auklets, a smell that is temporally elevated during the breeding season, may be a social odour that the birds use to recognise each other, for example by sniffing in each others' neck, where the smell is most apparent (Hagelin *et al.* 2003). One of the aldehyde compounds, decanal, that produce the citrus-like smell of crested auklets is known to be secreted by the preen gland, although the other aldehydes were not present in preen gland secretions (Douglas 2006).

Colour

Plumage colouration plays an important role in communication between birds in threat displays and mate choice (e.g. Burtt 1979, Andersson 1994, Delhey *et al.* in press), during which individual variation in feather colouration may signal individual quality. As feathers are dead structures and are replaced only infrequently during moult, the signal changes little with individual condition. Some bird species use cosmetic colouration as a more flexible visual quality signal, and dye

their feathers with endogenously produced cosmetics or cosmetics obtained from the environment (reviewed in Delhey *et al.* in press). Preen waxes have been described to be used as a cosmetic in hornbills (several genera) that elaborately apply coloured preen wax onto their plumage and casque that thereby change colour (Kemp 2001). Similar cosmetic plumage colouration during the breeding season only, has been found in brown pelicans *Pelicanus thagus* of which the yellow on the head becomes more intense after rubbing it on the preen gland (Schreiber *et al.* 1989). More – unsubstantiated – examples of the use of preen wax as avian cosmetics are given by Delhey *et al.* (in press).

Other functions

Cook *et al.* (2003) proposed that preen wax may protect birds' eggs against infection by fungi after the wax, actively or via the plumage, has been smeared onto the eggs. As the heat insulative qualities of (shorebird) nests are highly dependent on the nest material being damp or dry (Reid *et al.* 2002), transfering preen wax to the nest scrapes could increase the waterproofing effects of the nest, and thereby the insulation.

Chemical composition of preen wax: the chemists' approach

The composition of preen waxes varies among bird species and has been subject of study for already four decades, resulting in many detailed chemical characterisations of the preen wax mixtures of several bird species (for a review of many species see Jacob & Ziswiler, 1982). Chemical studies of preen wax composition are usually performed using gas chromatography (GC) - mass spectrometry (MS), mainly after the hydrolysis of the preen gland secretions. Only recently, more became known about the intact wax secretions (e.g. Dekker *et al.* 2000, Sinninghe Damsté *et al.* 2000, Burger *et al.* 2004, Haribal *et al.* 2005). Preen waxes of most species consist predominantly of wax esters, esterified alcohols and fatty acids (fig. 1.2). As the location and length of branching of the carbon skeleton varies, this results in very complex mixtures of waxes (e.g. Dekker *et al.* 2000). Most wax esters in preen wax secretions are monoesters of saturated, unbranched and/or mono-, di- or polymethyl branched carboxyclic acids which are esterified with alcohols, most often straight chain or methyl substituted mono-alcohols (e.g. Jacob 1979, Jacob & Ziswiler 1982). Next to monoester waxes, preen gland secretions may also comprise diester waxes, which can be divided in diol-based esters (diols esterified with two fatty acids, fig. 1.2) and diesters based on ß-hydroxy fatty acids usually at the 2 or 3 position, esterified with an alcohol and a fatty acid (fig. 1.2). Next to mono- and diesters, preen gland secretions of a few

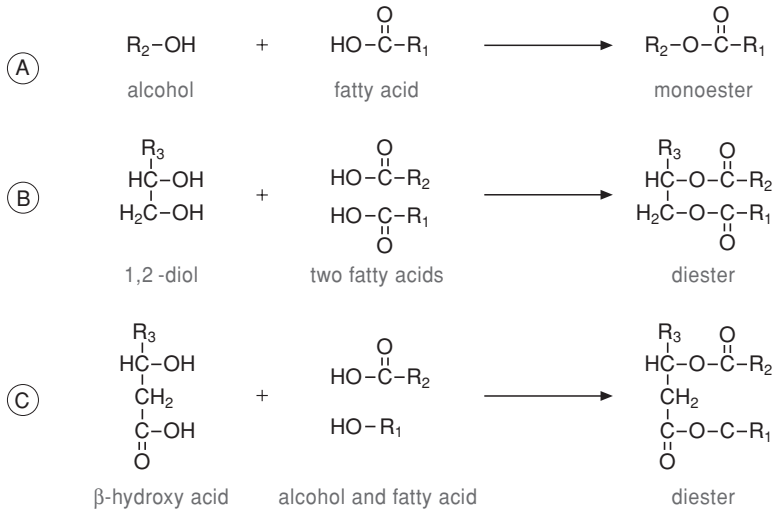

Figure 1.2 Schematic presentation with structural formulas of the esterification to (A) monoesters, (B) 1,2-diol-based diesters and (C) ß-hydroxy fatty acid based diesters.

species also contain triterpenoids and steroids such as squalane, cholesterol and cholestanone (Jacob & Ziswiler, 1982). However, some of these latter compounds may have been the result of contamination (e.g. Elder 1954). Preen wax mixtures thus mainly consist of rather stable, unsaturated, relatively large molecules. However, in many studies volatile components may have remained undetected (Haribal *et al.* 2005, Soini *et al.* unpubl. MS).

The fact that the secreted wax esters are composed of fatty acids and alcohols that not only vary in chain length but also in degree and location of branching of the carbon skeletons, results in complex mixtures of many different wax esters in preen gland secretions. Each bird species secretes a unique distribution of preen wax esters.

Variation in preen wax secretion has been used to generate phylogenetic hypotheses (mainly by J. Jacob, see Jacob 1992 for a review). This was a first careful step in the direction of biological interpretation of variation in preen wax composition. However, preen wax composition is likely the product of natural selection, and different bird species might have the same evolutionary answer to ecological problems, i.e. divergent or convergent evolution may take place and will bias phylogenetic relationships between species if based on preen wax compounds rather than on neutral molecular markers. Sweeney *et al.* (2004) argued that the use of single GC-MS peaks as independent data may flaw phylogenetic reconstructions because the same biochemical pathway can produce several different preen wax

molecules, resulting in covariation of different molecules within closely related species. A different approach, in which a proper detailed characterisation of the species-specific compounds of preen gland secretions in a phylogenetic context, may yield interesting hypotheses about the biological functions of the preen wax secretions, will bring us further in understanding variation in preen wax composition (cf. Sweeney *et al.* 2004, Haribal *et al.* 2005). This will be especially interesting when combined with experiments in which the function of specific compounds are being tested. Haribal *et al.* (2005), who studied the preen wax composition of several passerines living in different habitats in temperate regions, argue that ectosymbionts, such as lice and mites, are the main selective force that has led to the evolution of a wide variety of preen wax mixtures. Given the many functions that preen waxes (might) have, it is, however, likely that many other different environmental aspects have also shaped the great diversity in (species-specific) preen wax mixtures (cf. Sweeney *et al.* 2004). The functional significance of these differences have, as far as I know, never been investigated, but are a great challenge to (cooperations between) chemists and evolutionary ecologists.

Seasonal variation in preen wax composition of red knots: the biologists' and chemists' approaches integrated

Recent research on a long distance migrating, High Arctic breeding shorebird, the red knot, has shown that the chemical composition of preen waxes also varies intra-individually (Piersma *et al.* 1999, Dekker *et al.* 2000, Sinninghe Damsté *et al.* 2000). Drastic shifts from the usual monoester mixtures to a mixture consisting of diester preen waxes only, has been shown to take place in red knots just before the start of the breeding season (Piersma *et al.* 1999, Sinninghe Damsté *et al.* 2000). Similar seasonal changes in preen wax composition had been shown before for female, wild and domesticated mallards (Jacob *et al.* 1979, Kolattukudy *et al.* 1987). Piersma *et al.* (1999) proposed that diester preen waxes might alter the appearance of birds' plumage and act as a sexually selected quality signal during mate choice. The honesty of the signal would be guaranteed by the energetic and/or time costs associated with the shift to more viscous diester preen waxes that were presumed to be more difficult to apply under the low temperatures that prevail in the High Arctic.

The occurrence of intra-individual, seasonal changes in preen wax composition suggests different balances between costs and benefits of the production, secretion and use of mono- or diester preen waxes in different times of the year. Such seasonal variation offers a nice opportunity to investigate the functional aspects of variation in preen wax composition within a species. To understand the

evolution of variation in preen wax compounds we need to establish their functions, i.e. the costs and benefits of different mixtures of preen waxes. Costs and benefits can be expressed in terms of energy, nutrition and time or as a reproductive currency. Natural selection is expected to select against costly traits if there are no benefits involved that outweigh the costs of such traits. For example, diester preen waxes could be functional in attracting mates but perform less well in protecting plumage against ectoparasites than monoester preen waxes do. In this thesis costs and benefits of mono- and diesters in sandpipers (Scolopacidae) will be explored in a comparative and an experimental approach.

Outline of this thesis

In chapter 2 preen wax composition (mono- or diesters) is investigated, in a comparative approach, in the context of life cycle stages of 19 sandpiper species and the habitats they live in. In chapter 3 the focus is on the parental care system of different sandpiper species of which a possible association of diester preen wax secretion with the incidence of incubation is investigated. In chapter 4 the degree of flexibility of red knots to change preen wax composition is investigated by comparing annual cycles of preen wax composition in both free-living and captive red knots and by experimentally perturbing the annual cycles by changing light regimes and the daily food availability. In chapter 5, the hypothesis of Piersma *et al.* (1999) reading that the shift to diester preen waxes in red knots serves as a temporal individual quality signal that changes the colouration of the plumage is tested. Spectrophotometry is used to measure the change in plumage colouration when preen wax composition changed from mono- to diesters and to measure the effect on plumage colouration of the absence or presence of preen wax on the plumage. Additionally, the light absorbance of isolated samples of preen wax is measured to investigate whether the (shift in) preen wax functions as a (temporal) sun blocker that protects feathers from damaging ultraviolet light. In chapter 6 the effects of natural amounts of mono- and diesters on feathers on the possible growth-inhibition of a feather-degrading bacterium, now known to occur in red knots, are tested. In chapter 7 it is experimentally investigated by use of a sniffer dog, whether the change in preen wax composition may olfactorily camouflage birds when they are incubating clutches that are easily accessible to mammalian olfactory-searching predators. Finally, in chapter 8 the main findings are summarised and placed in the broader context of variation of preen wax composition, the cost and benefits of -production, secretion and application of- different preen wax compounds are discussed in this chapter and suggestions for future research provided.

Box A Preening behaviour of red knots: time budgets and use of preen wax

Jeroen Reneerkens & Maaike A. Versteegh

The preen gland is omnipresent in almost all bird species. It is clear that the secreted waxes from the gland play a biological role on the feathers, where the waxes are spread onto with the birds' bill during daily maintenance activities. We studied the preening behaviour of seven captive red knots to get a better view on the time that is daily devoted on preening behaviour, especially with respect to the actual application of preen wax onto the feathers. Furthermore, we wanted to find out on which parts of the plumage birds smear preen wax and whether this is equal for each of the plumage parts.

Methods

Observations of an hour were done on 26 April, 24, 29, 31 May and 4 and 6 June 2002 at a random time during the daylight period (six hrs of observation in total, seven birds). The birds were studied through a small window and never disturbed by the observers. The ground surface of the outdoor aviary in which the red knots were housed, measured 2 m by 4 m. It contained a small mudflat with flowing salt water and a tray with streaming fresh water was available for bathing and drinking. Food (trout pellets) were available *ad libitum*.

Gas chromatograms of the preen waxes sampled on each day of observation showed that most birds secreted monoester B preen wax at 26 April, but all gradually changed to diesters during the following days of observation secreting a mixture of monoesters B and diesters and pure diesters during the last days in some birds. We did not collect enough observations on red knots secreting pure diester preen waxes to have enough statistical power to find possible differences in preening behaviour with chemical composition of the preen wax.

All behaviour was filmed with a small digital video camera so that all behaviour could be studied in much more detail afterwards. The birds were ringed with individually unique colour codes, enabling individual recognition. We analysed the behaviour afterwards using The Observer 3.0 Event Recorder (Noldus Information Technology, Wageningen, The Netherlands) which allowed us to precisely measure the duration of all events.

Every preening and bathing event was scored and the duration of each bout was measured to the tenth of a second. Preening was defined as the active behaviour during which a bird uses its bill to distribute preen wax onto feathers or to rearrange the feathers. As preen wax is applied onto head feathers by rubbing the head directly over the preen gland, and thus does not involve the use of the bill, this behaviour was scored as preening behaviour too. A preening bout was defined as preening behaviour that was not interrupted. This meant that when a bird stopped preening for a moment, often but not always followed by preening a different part of the plumage, a bout was ended. Bathing was included as it involves feather maintenance too and often some preening occurred during bathing. The duration of preen gland massage with the bill, to collect waxes onto the bill for distribution onto the plumage, was carefully measured to the tenth of a second. The length of the following preening bout, as well of preening bouts that did not involve preen wax application, was measured.

We scored on which body parts the collected waxes were applied. Because the birds usually continued preening after preen wax harvesting on its bill for sometimes half an hour or more, it was often difficult to judge when preen wax application stopped and feather rearrangement started. Therefore, we only scored the first body part to which the wax had been applied after the bird touched the preen gland with its bill. We distinguished the abdomen, breast, head, back, tail and wings as different body parts.

Results

The birds spent on average 7.2% of the time preening (range 5.0-11.2 %). During the 6 hrs of observation we recorded 165 times that preen wax was collected from the gland (average 23.6 times per bird, range 5 – 49). On average, 2.9% of all preening bouts involved application of preen wax. This is similar to 3.1% found in free-living barn swallows (Møller 1991). The preen gland was touched for wax harvesting 4.0 hr^{-1} (range 0.7 – 7.0 hr^{-1}) and lasted on average 5.1 sec hr^{-1}. Preening bouts following wax collection lasted on average 1 min 13 sec (n= 142). Average preening bouts differed between individuals (ANOVA $F_{4154,6} = 4.448$, $P<0.001$) and those preening bouts that did not involve wax application lasted on average 42 sec longer (1 min 55 sec; ANOVA $F_{4154,1} = 3.854$, $P=0.05$). The interaction term was not significant and removed from the test.

Although there was some variation in the frequency of preen wax application on the different parts of the plumage, this was not significant (fig. A1A, ANOVA $F_{36,5} = 0.465$, $P = 0.800$). The pattern that the birds more often preened the smaller parts of the plumage (abdomen, back and head, fig A1A) seemed reversed in the duration of such preening bouts (fig. A1B),

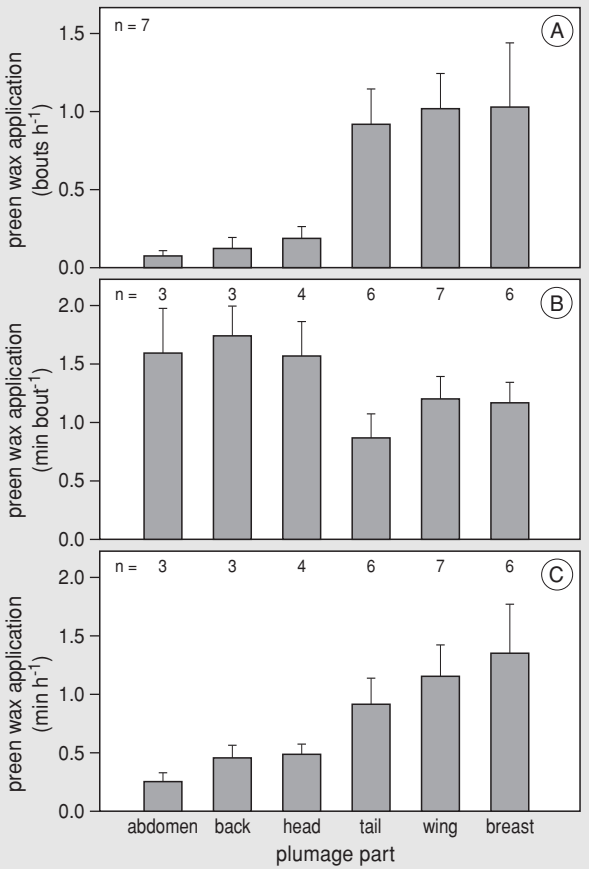

Figure A1 The average number per hr that preen wax was applied to different parts of the plumage by seven captive red knots (A). In (B) the duration of the application of wax on these parts of the plumage is depicted (of those individuals in which bouts of preen wax application to particular parts of the plumage were observed). The product of (A) and (B) -again for those individuals in which a given bout of preen wax application was observed- is depicted in (C) and represents the total amount of time spent on applying preen wax onto a given part of the plumage.

but this was also not statistically significant (ANOVA $F_{23,5}$= 2.307, P= 0.142). A post-hoc analysis (Fisher's least significant difference test) showed, however, that bouts during which the tail was preened lasted significantly shorter than those bouts during which other parts of the plumage were preened (P<0.05, except for the difference with abdomen: P=0.055). Nevertheless, the total amount of time spent preening wax (the product of the number of bouts hr^{-1} and the duration of these bouts) was equal for all parts of the plumage (fig. A1C ANOVA $F_{23,5}$ = 1.955, P= 0.124).

Discussion

We can conclude that preen wax is smeared on all parts of the plumage. The individual time bouts during which preen wax was applied onto the tail lasted on average a bit shorter compared with the other parts of the plumage, but this difference was absent in the frequency of preen wax application and the total time per hr spent preening. The distribution of preen wax over the different plumage parts, as depicted in fig. A1A, may be biased if the birds have a certain preening routine during which a certain plumage part (e.g. the breast feathers) is always preened first after wax collection from the preen gland. During a second preening bout following contact with the gland, there might still be wax left on he bill that is spread onto (a different part of) the plumage, but because we could not be sure of that, this was not counted as preen wax application in our study. Only a small amount of all time spent preening involves the application of waxes onto the feathers. It will be interesting to find out whether preening behaviour changes with the chemical composition of the wax. Piersma *et al.* (1999) suggested that diester preen waxes would be more difficult to smear than monoester preen waxes due to their higher viscosity, especially in the cold arctic environment during which diesters are secreted under natural conditions. The captive red knots secrete diester preen waxes during summer in The Netherlands (chapter 4), when ambient temperatures are usually high, in contrast to the arctic summers experienced by free-living red knots. Given the little time daily spent on preen wax application, we do not expect that a change in preen wax composition entails high costs in terms of time.

Acknowledgements
We thank Bernard Spaans for taking care of the birds and for being careful not to cause any disturbance during our observations.

CHAPTER 2

Sandpipers (Scolopacidae) switch from monoester to diester preen waxes during courtship and incubation, but why?

Jeroen Reneerkens, Theunis Piersma & Jaap S. Sinninghe Damsté

Proceedings of the Royal Society of London: Biological Sciences 269: 2135–2139

ABSTRACT

Recently, a shift in preen wax composition, from lower molecular weight monoesters to higher molecular weight diesters, was described for individuals of a sandpiper species (red knot, *Calidris canutus*) that were about to leave for the tundra breeding grounds. The timing of the shift indicated that diester waxes served as a quality signal during mate choice. Here, this hypothesis is evaluated on the basis of a survey of preen wax composition in 19 sandpiper species. All of these species showed the same shift observed in the high-Arctic breeding red knots. As the shift also occurred in temperate breeding species, it is not specific to tundra-breeding sandpipers. Both sexes produced the diester waxes during the incubation period until hatching, in addition to the short period of courtship, indicating that diesters' functions extend beyond that of a sexually selected 'make-up'. The few non-incubating birds examined (males of curlew sandpipers (*C. ferruginea*) and ruffs (*Philomachus pugnax*)) had the lowest likelihood of secreting diesters, indicating a functional role for diester preen waxes during incubation. We propose that diester preen waxes enhance olfactory crypsis at the nest.

Introduction

A complete and abrupt shift has recently been discovered in the chemical composition of secretions from the uropygial gland (preen gland) in high-Arctic breeding red knots (*Calidris canutus*) (Piersma *et al.* 1999). Although these secretions were previously considered invariable and taxon-specific (Jacob & Ziswiler 1982), a rare class of diesters (Sinninghe Damsté *et al.* 2000) completely replaces the usual mixture of monoesters at the start of courtship in this species.

The uropygial gland secretions are preened into the plumage (hence the name 'preen waxes') and several functions have been proposed (Jacob & Ziswiler 1982). Preen waxes may delay feather wear, keep feathers flexible (Stettenheim 1972) and waterproof (Elder 1954; but see Fabricius 1959; Elowson 1984) and have anti-dermatophytic characteristics (Jacob *et al.* 1997). This compositional shift indicated an additional function for diester preen waxes during the period of courtship and mating (Piersma *et al.* 1999). Diester preen waxes are more viscous than monoester mixtures and they may be difficult to preen into the plumage under the prevailing cold temperatures during the summer season in the high Arctic. The change to a preen wax mixture that brings about additional costs led Piersma *et al.* (1999) to propose that diester preen waxes may function as a sexually selected quality signal, perhaps by enhancing the appearance or reflectance of the plumage.

In this study, we explore the idea that diester preen waxes function as a sexual signal. We do so in a comparative way by studying the chemical composition of preen gland secretions before, during and after the reproductive period in 19 closely related sandpiper species of the Charadriiform family Scolopacidae.

Methods

Birds

Sandpipers were caught at various stages of their annual cycle and preen wax samples were collected. All investigated species are migratory and use different areas for reproduction and wintering, often thousands of kilometres apart. Except for black-tailed godwits (*Limosa limosa*), redshanks (*Tringa totanus*), Asian dowitchers (*Limnodromus semipalmatus*) and some of the ruffs (*Philomachus pugnax*) that breed in temperate regions, all investigated sandpipers reproduce on the (sub-) Arctic tundra (table 2.1). They typically winter in (sub-) tropical or temperate coastal salt-water habitats (Piersma 1997). To reach the Arctic breeding areas between late May and early June, sandpipers make long-distance flights of thousands of kilometres, with one or two intermediate refuelling stops in wetland habitats (summarized in Piersma *et al.* 1996).

Table 2.1 Frequencies of diester preen waxes that individuals of 19 sandpiper species secrete during spring migration, pre-breeding, incubation, chick guarding, autumn migration and during winter. Individuals that secreted mixtures of monoesters and diesters were scored as 0.5.

	species		adults						juveniles
subfamily	common name (scientific name)	breeding range	spring migration	pre-breeding	incu-bation	chick guarding	autumn migration	winter	post-fledging, breeding grounds
godwits	black-tailed godwit (*Limosa limosa*)	temperate	1/10		11/11	2/2			
	Hudsonian godwit (*Limosa haemastica*)	low Arctic	0/3		1/1				
	bar-tailed godwit (*Limosa lapponica*)	high Arctic	0.5/34	1/1			0/26	0/9	
shanks	redshank (*Tringa totanus*)	temperate	0/7		45/48	0.5/2	0/4		
turnstones	ruddy turnstone (*Arenaria interpres*)	high Arctic	0/40	10.5/12	15/15	0.5/2	0/19	0/3	0/6
phalaropes	red phalarope (*Phalaropus fulicarius*)	low and high Arctic			1.5/2		0/1		0/3
dowitchers	Asian dowitcher (*Limnodromus semipalmatus*)	temperate			1.5/2			0/4	
	short-billed dowitcher (*Limnodromus griseus*)	low Arctic	0/2		3/3				
sandpipers	red knot (*Calidris canutus*)	high Arctic	0.5/65	24/26	14.5/15	0/3	0/33	0/9	0/8
	sanderling (*Calidris alba*)	high Arctic	0.5/20	2/3	4/4		0/18		
	semipalmated sandpiper (*Calidris pusilla*)	low and high Arctic	0/6				0.5/1		
	western sandpiper (*Calidris mauri*)	high Arctic	0/4		32.5/33				
	little stint (*Calidris minuta*)	high Arctic	0/2	11/12	11/11	0.5/1	0.5/19	0/10	0/13
	Temminck's stint (*Calidris temminckii*)	low and high Arctic			2/2			0/2	
	white-rumped sandpiper (*Calidris fuscicollis*)	high Arctic	0/9		3/4				0/16
	Baird's sandpiper (*Calidris bairdii*)	high Arctic			16.5/17				0/2
	dunlin (*Calidris alpina*)	low and high Arctic	0.5/14	5/7	11/11	0.5/1	0/14	0/8	0/7
	curlew sandpiper (*Calidris ferruginea*)	high Arctic		11.5/20	6/6	0/2	0/20	0/5	0/8
	ruff (*Philomachus pugnax*)	temperate-high Arctic	10/92					0/9	
all species			13/308	65/81	177.5/185	4/13	1/155	0/59	0/63
percentage			4	80	96	13	0	0	0

Sex and life-cycle stage

The composition of preen wax secretions was studied in relation to sex and life-cycle stage. We determined the sex of the birds by examining sex-specific plumage traits, size differences and/or sex-specific behaviour (e.g. incubation in some of the species). The sexually monomorphic red knots were sexed using a standard and verified molecular technique (Baker *et al.* 1999). Most sanderlings (*C. alba*), semipalmated (*C. pusilla*), Baird's (*C. bairdii*) and white-rumped sandpipers (*C. fuscicollis*) were not sexed individually. Sexes of red phalaropes (*Phalaropus fulicarius*), Hudsonian godwits (*L. haemastica*) and bar-tailed godwits (*L. lapponica*) could not be compared, as a single individual or only one of the sexes was caught during courtship and incubation.

Birds caught shortly after arrival on the Arctic breeding grounds were considered to be in the period of mate choice and courtship. High-Arctic breeding sandpipers start courtship displays within a few days of arrival (e.g. Reneerkens *et al.* 2002). Sometimes they arrive already paired-up, as observed in some of the curlew sandpipers (H. Schekkerman & I. Tulp, pers. comm.). Birds on the breeding grounds with fully developed brood-patches were considered to be incubating even if not caught on the nest.

In redshanks and western sandpipers (*C. mauri*), it was possible to relate the composition of preen-gland secretions to the number of days before hatching. Hatching dates were calculated from known laying dates, using the incubation lengths (24 days and 19 days, respectively) measured at the study sites (A. Niehaus & W. Tijssen, pers. comm.).

Sample processing

By softly massaging the nipple of the preen gland, a tiny sample of preen wax can be obtained on a cotton bud. The waxes were dissolved in ethyl acetate. We then evaporated the solvent with a gentle flow of nitrogen gas and weighed the waxes (ranging from 0.1–4.3 mg). Subsequently, the waxes were redissolved in ethyl acetate to a concentration of 1 mg ml^{-1} and injected into a gas chromatograph (GC). Details of the analytic procedures are described elsewhere (Dekker *et al.* 2000). Gas chromatograms of the wax mixtures are characteristic for either mono- and diesters (Piersma *et al.* 1999; Sinninghe Damsté *et al.* 2000) enabling easy classification of samples into three groups: (i) monoesters, (ii) diesters and (iii) a mixture of mono and diesters (fig. 2.1). This classification was confirmed by GC and by GC followed by mass spectrometry analysis of hydrolysed waxes (cf. Dekker *et al.* 2000). We scored the fraction of diesters in the preen waxes as: 0, only monoesters; 0.5, mixture of mono- and diesters; and 1, predominantly (more than 95%) diesters.

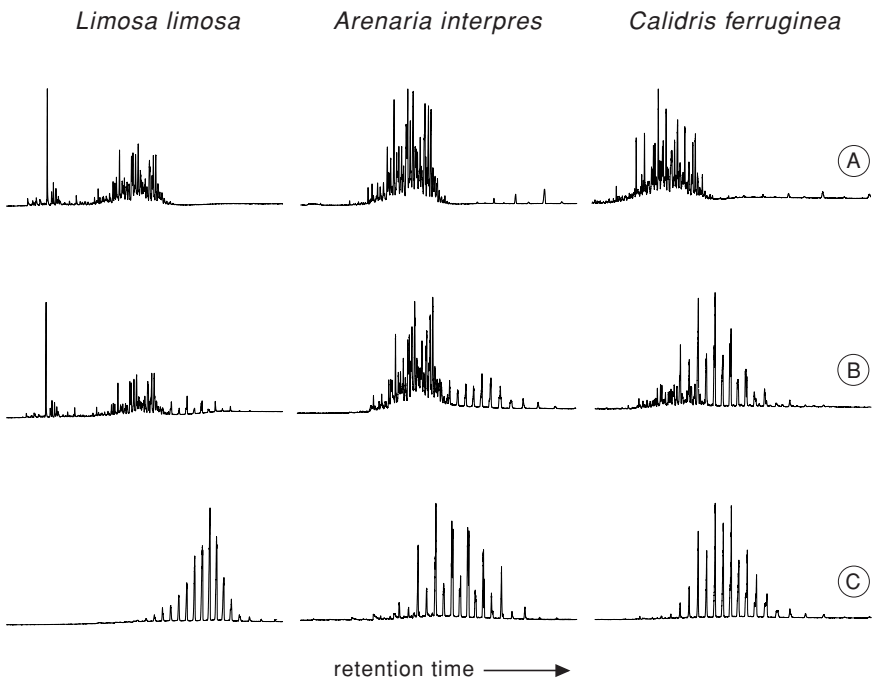

Figure 2.1 Gas chromatograms of typical (A) monoester, (B) mono/diester, and (C) diester secretions of black-tailed godwit (*Limosa limosa*), ruddy turnstone (*Arenaria interpres*) and curlew sandpiper (*Calidris ferruginea*).

Results

During migration and in winter, all 19 investigated sandpiper species secreted mixtures of monoester preen waxes. As in red knots (Piersma *et al.* 1999), shifts from mono- to diester waxes only occurred at the start of courtship and mating (table 2.1; fig. 2.2).

Shortly before departure to the breeding grounds (usually late May), diester waxes were produced by a few individuals. By contrast, shortly after arrival on the breeding grounds, the majority of individuals (80%) secreted diesters (table 2.1). This indicates that the shift in preen wax composition occurs around arrival on the Arctic tundra. Almost all individuals (96%) produced diesters during incubation (table 2.1). Only a few incubating redshanks and western sandpipers secreted (some) monoester compounds five days or less before hatching. However,

most adults with chicks secreted monoester preen waxes (table 2.1), indicating a sharp shift from diesters to monoesters at hatching. During autumn migration and winter preen gland secretions never consisted of diesters (table 2.1). Recently fledged juveniles (63 individuals of eight species) only secreted monoester waxes (table 2.1).

Redshanks showed more overlap in the temporal pattern of mono- and diester secretion than other species (fig. 2.2). This is caused by the temporal overlap of life-cycle stages (table 2.1). Where verifiable, diester secretion during courtship and incubation occurred evenly in both sexes. However, curlew sandpipers and ruffs were different. In curlew sandpipers, only two of the 10 male curlew sandpipers secreted complete diester mixtures shortly after arrival on the breeding grounds. By contrast, eight of the 10 females from this period secreted diester waxes only (Mann–Whitney U-test with tied ranks, $U_{1,20} = 82.0$, $P = 0.008$). Of the 49 male and 43 female ruffs caught during spring migration (table 2.1), none of the males and 14 of the females produced (mixtures with) diesters (fig. 2.2). All captive female (and no male) ruffs sampled during the period of incubation secreted diester preen waxes (chapter 3).

Discussion

How common are changes from mono- to diester preen waxes?
Jacob & Poltz (1973) and Jacob (1978) characterized preen wax components of seven shorebird species, including three of the species that we investigated (redshank, red knot and dunlin) and found monoesters with some traces of diesters. It is not known when their samples were taken. In this study, we demonstrated that in all 19 species examined, during the short period of mate choice and incubation, wax composition shifted from mono- to diesters. As temperate breeding species start their reproductive activities earlier than high-Arctic breeding species, diesters are secreted earlier in temperate breeding species (black-tailed godwit, redshank and ruff; fig. 2.2).

What function(s) do diester preen waxes serve?
In all 19 sandpiper species, diester waxes are secreted during the relatively brief periods of courtship and incubation, indicating a common function shaped by the specific demands during these life-cycle stages. Because adults (as well as juveniles) of Arctic breeding species excrete monoesters after hatching, Arctic conditions (e.g. low temperatures, strong winds and high ultraviolet radiation) are unlikely to have selectively favoured diester preen waxes as a way of plumage protection. As they also occur in redshanks, black-tailed godwits and Asian dowitchers,

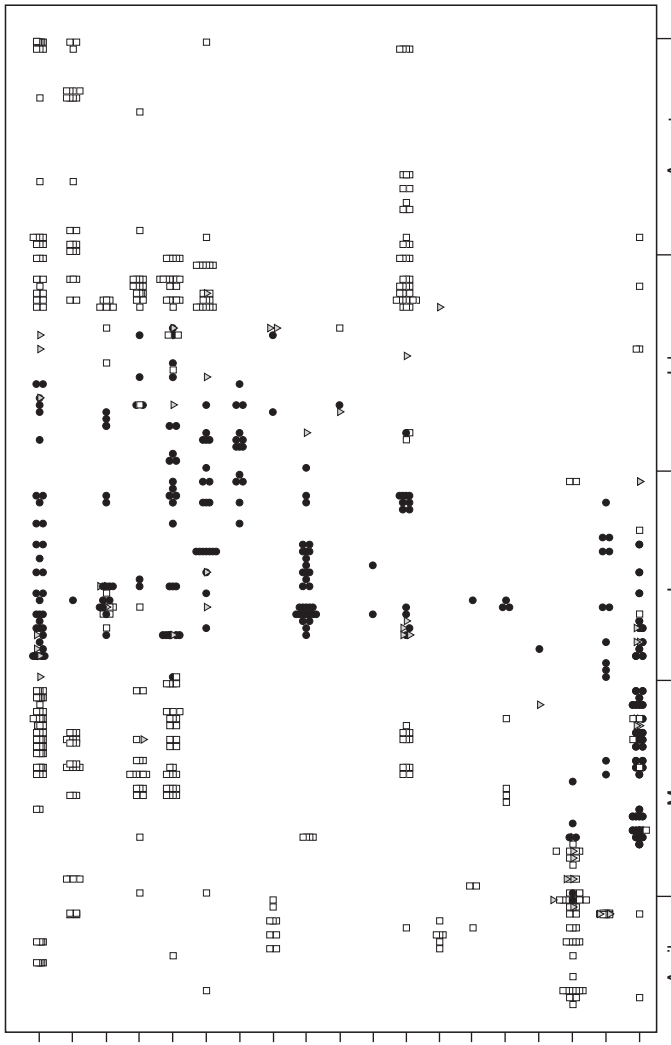

Figure 2.2 Seasonal changes in chemical composition of preen waxes of adult birds in 19 sandpiper species. Species are ordered from top to bottom on the basis of median lattitude of their breeding range, with the northernmost breeding species first. Squares, monoesters; triangles, mixtures of mono- and diesters; filled circles, diesters.

shifts to diesters are not restricted to Arctic breeders. The more distantly related oystercatcher (*Haematopus ostralegus*, Haematopodidae) breeds and overwinters in western Europe and also shifts to diester preen waxes during the breeding season (J. Reneerkens, unpublished data).

Ruffs and curlew sandpipers are, to our knowledge, the only two investigated sandpipers in which incubation is completely or largely restricted to females. Secretion of diester waxes is also restricted to female ruffs and occurs significantly more often in female than in male curlew sandpipers. In wild-type and domesticated mallards (*Anas platyrhynchos*), females, but not males, show similar qualitative shifts from mono- to diester preen waxes during courtship and incubation (Jacob *et al.* 1979; Kolattukudy *et al.* 1987). In mallards, incubation is also restricted to females. The change to diester wax secretion in incubating individuals indicates that diesters are important for birds on the nest. Diester preen waxes have higher molecular weights than monoesters and consequently are less volatile. Thus, they may reduce the smell and enhance olfactory crypsis. If diesters make it more difficult for mammalian predators, such as Arctic foxes (*Alopex lagopus*), to smell out the bird on the nest, a shift from mono- to diester preen waxes during incubation would have a large selective advantage.

Piersma *et al.* (1999) proposed that diesters enhance sexually selected quality signals that make the plumage brighter or shinier, enabling visual discrimination of fit mates during mate choice. The present study does not falsify this hypothesis, but indicates that it is not the whole story because diester preen waxes are also secreted during incubation, that is, after fertilization. Small differences in the smell or in the visibility of plumage due to different wax compositions may not be detectable by the human senses (e.g. Viitala *et al.* 1995). However, if different preen wax compositions can be distinguished by conspecifics, they could potentially play a part during mate choice before becoming fully functional during incubation.

Acknowledgements
We thank our NIOZ colleagues Anne Dekinga, Maurine Dietz, Graciela Escudero, Petra de Goeij, Anita Koolhaas, Luisa Mendes and especially Bernard Spaans, for their help with bird catching and preen wax sampling on locations worldwide. Leo Bruinzeel, Nicola Baccetti, Welmoed Ekster, Frank Engelen, Adrian Farmer, Katharine Graham, Niko Groen, Tómas Gunnarsson, Joe Jehl Jr, Joop Jukema, Jan van der Kamp, Tatyana Kirikova, Joanna Klima, Oscar Langevoord, Dov Lank, Åke Lindström, Ron Mes, Guy Morrison, Amanda Niehaus, Leon Peters, ringing group 'Calidris', Castricum ringing group, Daniel Ruthrauff, Hans Schekkerman, Mikhail Soloviev, Adriano Talamelli, Wim Tijssen, Ingrid Tulp, Douwe van der Zee and several anonymous contributors also provided preen wax samples. J.R. thanks the Canadian Forces for their support at the military station at Alert and the Canadian Wildlife Service for financial and logistic support of work in 1999, and Jack Stephens and Kurt Burnham of the Peregrine Fund for hospitality in Thule, Greenland. John Wingfield is acknowledged for enabling J.R. to

participate in fieldwork in Thule. Muriël van den Anker and Soledad van Eijk helped in the laboratory. We thank Pavel S. Tomkovich, Ingrid Tulp, Hans Schekkerman and Dov Lank for early feedback on the results, and Phil Battley, Sue Moore and an anonymous referee for improving an earlier draft. The work is financially supported through the Netherlands Organization for Scientific Research (NWO) by ALW grant 810.34.003 and a PIONIER-grant to T.P. This is NIOZ publication 3700.

Box B Compositional differences in diester preen waxes of ecological contrasting species within three shorebird families

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Birds apply waxes secreted from their uropygial gland, also called preen gland, onto their feathers during plumage maintenance. The function of these preen waxes has been subject of scientific discussions for many decades. Suggested functions include waterproofing the plumage (although clear evidence is lacking: e.g. Elder 1954, van Rhijn 1977), reduction of feather abrasion and influencing the microbial flora in birds' plumages (Shawkey *et al.* 2003, chapter 6). In some bird species preen wax also has a cosmetic function (Delhey *et al.* in press). Preen gland secretions are complex mixtures consisting of many components -mainly monoesters (alcohols condensed with fatty acids)- that are species specific (Jacob & Ziswiler 1982, Sweeney *et al.* 2004). Detailed descriptions of preen wax composition have been used in phylogenetic and taxonomic contexts (e.g. Jacob 1976, 1978, Jacob & Poltz 1973, Jacob & Ziswiler 1982, Sweeney *et al.* 2004). However, recent studies indicate that preen wax composition may change seasonally and between sexes (e.g. Jacob *et al.* 1979, chapters 2, 3 and 4). At least 19 sandpiper species (Scolopacidae: chapter 2) and six plover species plover (Charadriidae: Reneerkens *et al.* 2006) exhibit seasonal changes in preen wax composition. These shorebirds secrete monoester preen waxes during the non-breeding period and incubating individuals of these species shift to diester preen waxes during the breeding period (chapter 3). Next to the complete shift from mono- to diester preen waxes, there is also seasonal variation in preen wax composition during the non-breeding season. Red knots *Calidris canutus* (chapter 4), ruffs *Philomachus pugnax* and other sandpiper species (chapter 3) secrete two different mixtures of

monoesters during distinct periods in the year. In previous studies in which preen wax composition of sandpipers has been used for taxonomic statements (Jacob & Poltz 1973, Hoerschelmann & Jacob 1992), the dates of preen wax sample collection have not been reported. Obviously, seasonal variation in preen wax composition may confound taxonomic and/or phylogenetic judgements based on preen wax composition.

Variation in preen wax composition is expected to be under sexual and/or natural selection pressures. To better understand variation in preen wax composition, both within (chapter 2) and between species, detailed and complete descriptions of all preen wax components are necessary. In this study we provide such description of the diester preen wax components of six species of shorebirds belonging to three families (sandpipers, godwits and plovers). Within each family two species with contrasting ecologies were selected. One species within each family breeds at high latitudes in the High Arctic and spends the non-breeding season almost strictly in saltwater habitats while the other species breed at lower latitudes and occur in freshwater habitats during the non-breeding season. These year-round habitat differences between species involve physical aspects (e.g. wind exposure, salinity), food type, migration distances, disease risk and genetic variability (Piersma 1997, 2003, Mendes *et al.* 2005).

If similarities in diester preen wax composition are greater between species of similar ecological niches than between species within each family, this might imply that certain environmental aspects of the saltwater/High Arctic and the freshwater/lower Arctic habitats selectively favour certain types of preen wax compounds. If, on the other hand, similarities within each family are greater than between species with similar ecologies, this might mean that preen wax composition is evolutionary conserved and not so much the result of current selection pressures. In this box, we present a general method to study which natural selection pressures might play a role in the shaping of preen wax composition.

Material and Methods

Birds

Preen wax samples were obtained from two species of calidridine sandpipers (*Calidris*; red knot *C. canutus* and little stint *C. minuta*), two godwits (*Limosa*; bar-tailed godwit *L. lapponica* and black-tailed godwit *L. limosa*)

and two plovers (*Pluvialis*; grey plover *P. squatarola* and Pacific golden plover *P. fulva*). Within the three families both a 'saltwater species' (red knot, bar-tailed godwit and grey plover) or a 'freshwater species' (little stint, black-tailed godwit and Pacific golden plover) were chosen. All birds were caught on their nest during incubation. Preen wax samples of a red knot were obtained of an individual near Alert, Ellesmere Island, Canada, belonging to the subspecies *islandica* on 20 June 1999, of a little stint (23 June 2002), bar-tailed godwit (26 June 2006), Pacific golden plover (23 July 2002) and grey plover (20 July 2003) on the tundra of the Taimyr penninsula, Siberia, Russia. The black-tailed godwit was sampled on Iceland on 11 June 2001.

To make sure that preen wax composition of individual birds of a certain species that breed at the higher or lower latidudinal extremes of their breeding range do not adapt to the local conditions, we compared diester preen wax sampled from a black-tailed godwit on Iceland (subspecies *islandica*) with a incubating conspecific caught in the Selenga delta, Russia, of the subspecies *melanuroides*.

Experimental procedures

Wax samples were taken from the breeding birds by softly massaging the preen gland with a cotton bud. The intact waxes were extracted with ethyl acetate and analysed by GC and GC-MS. For transesterification of the diester waxes (Kolattukudy *et al.* 1987) about 1 mg was solved in 300μl toluene and 1.5 ml of 14% BF3 in methanol was added and the mixture was refluxed for 3 h. After cooling, 2 ml of bidestilled water was added and the mixture was extracted with DCM and dried over Na_2SO_4. The produced fatty alcohols and hydroxy fatty acid methylesters were converted to their trimethylsilyl derivatives by heating with BSTFA/pyridine at 60°C for 20 min and the mixture was diluted with ethyl acetate and analysed by GC and GC-MS. The ratio of the diols to hydroxy fatty acids is based on integration of the GC peak areas, corrected for the trimethyl silyl groups in case of the diols and the trimethyl silyl group and the methyl group for the hydroxy fatty acids taking into account the molecular weights of the underivatised compounds.

GC and GC-MS

GC was performed on a Hewlett-Packard 5890 series II chromatograph equipped with an on-column injector and fitted with a 25 m x 0.32mm fused silica capillary column coated with CP-Sil 5 (film thickness 0.12 um).

Helium was used as carrier gas. For the intact waxes the oven was programmed from 70 to 130°C at 20°C min^{-1}, followed by an increase of 4°C min^{-1} to 320°C and held at 320°C for 35 minutes. For the transesterified fractions the oven was hold on 70°C for 3 min and programmed to 320°C with 4°C min^{-1}. Compounds were detected using a flame ionization detector (FID). GC-MS was performed on a Thermofinnigan Trace gas chromatograph using the same column and conditions as described for GC. The column was directly inserted into the electron impact ion source of a Thermofinnigan DSQ quadrupole mass spectrometer, scanning a mass range of *m/z* 50-800 at 3 scans s^{-1} and an ionization energy of 70 eV.

Similarity analysis

We calculated Bray-Curtis similarity coefficients (Bray & Curtis 1957) to compare similarities between the chemical composition of the preen wax secretions of the six species, based on the relative amounts of the products of transesterification of diester preen waxes. The relative amounts were estimated by integration of the GC-MS peaks which were corrected for mole mass and the addition of carbon resulting from derivatization

Results

The carbon chain lengths of the diester preen waxes of all six species ranged roughly between C_{30} and C_{54}. The relative abundance of different components differed per species resulting in characteristic gas chromatograms of the intact waxes for each species (fig. B1). For example, the highest peaks in the gas chromatograms were species specific (fig. B1, table B1). Although the range of the total number of carbon atoms of the secreted diester preen waxes are similar for the six shorebird species, mass spectrometric analyses before and after transesterification showed that the chemical composition of the preen waxes differed greatly. It is clear that the molecular composition of diester preen wax is also species specific, like monoester preen wax (e.g. Jacob & Poltz 1973, Hoerschelmann & Jacob 1992). The chemical composition of the secreted diesters by black-tailed godwits from Iceland and Russia showed that both individuals secreted the same diester compounds. This suggests that there exists little inter-individual variation in preen wax composition within species. A previous study of many gas chromatograms of intact mono- and diester preen wax of red knots (chapter 4) also suggested little

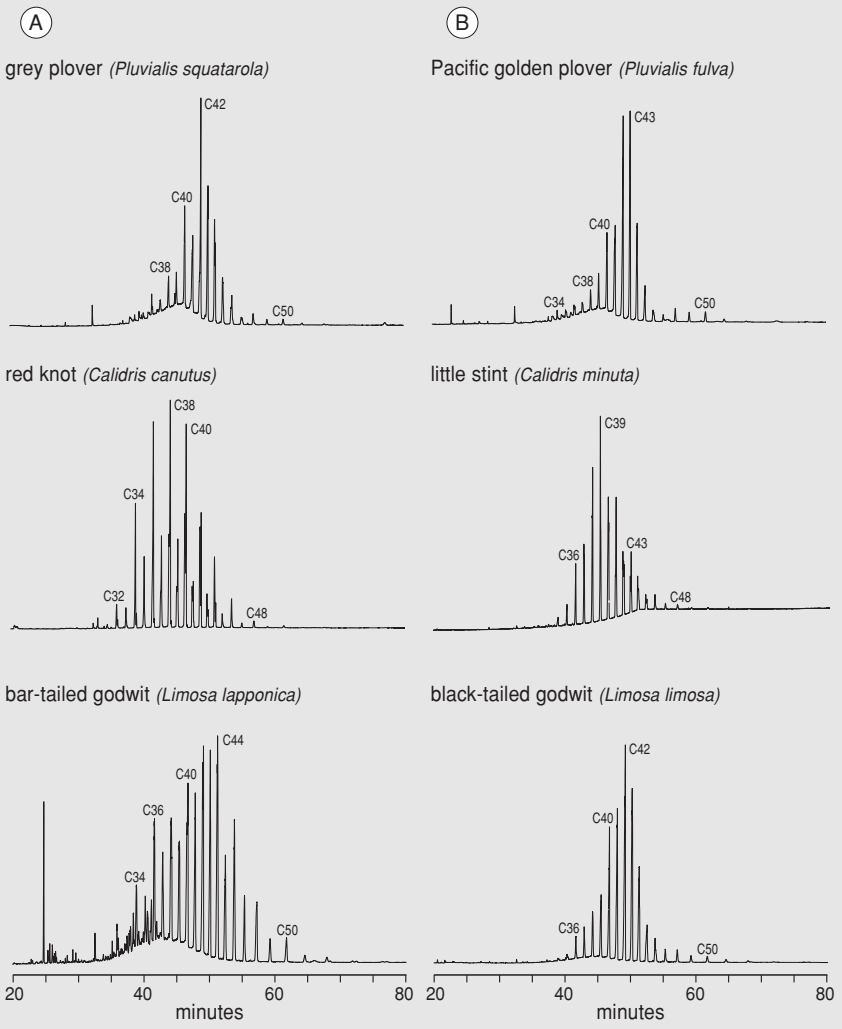

Figure B1 Gas chromatograms of the intact diester preen wax mixtures of six shorebirds. The saltwater/High Arctic species are depicted in (A), the fresh water/subarctic species in (B).

inter-individual variation in preen wax composition. The relative amounts of the components were, however, different for the two subspecies of black-tailed godwit.

Table B1 Comparison of diester preen wax composition between six shorebird species. Range refers to the range in carbon chain lengths of the intact diester waxes, the maximum to the highest relative fraction. See text for the methods of calculating the percentage of diesters composed of 1,2-diols and ß-hydroxy fatty acids.

	red knot	little stint	bar-tailed godwit	black-tailed godwit	grey plover	Pacific golden plover
	Calidris canutus	*Calidris minuta*	*Limosa lapponica*	*Limosa limosa*	*Pluvialis squatarola*	*Pluvialis fulva*
Range	C_{30}–C_{50}	C_{33}–C_{52}	C_{34}–C_{52}	C_{33}–C_{52}	C_{34}–C_{52}	C_{34}–C_{52}
Maximum	C_{38}	C_{39}, C_{40}	C_{42}, C_{43}	C_{38}-C_{46} even	C_{42}	C_{42}
%						
1,2-diols	82	86	60	20	21	23
β-OH-FA	18	14	40	80	79	77

The diester preen waxes of the six species consists of two structurally different kind of diesters (fig B2); those that are based on 1,2-diols esterified with two straight chain fatty acid molecules (cf. Sinninghe Damsté *et al.* 2000), and those that are based on β-hydroxy fatty acids esterified with a straight-chain fatty acid and a straight-chain alcohol (fig. B2, table B1; also see fig. 1.2). The preen wax of the sampled bar-tailed godwit did not only consist of diesters, but also contained some monoesters, presumably because the wax composition was was being transformed back to pure monoesters at the end of incubation (cf. chapter 2).

The relative amount of all diesters that are based on 1,2-diols was large in the sandpipers (82-86%) and low in the plovers (21-23%; table B1). Preen wax of the sandpipers contained C_{12}-C_{20} 1,2-diols, whereas the plovers and godwits only possessed diesters based on C_{15}-C_{20} 1,2-diols. The two godwit species differed in the fraction of diol-based diesters relative to diesters based on β-hydroxy fatty acids. The percentage of 1,2 diols is 20% in the black-tailed godwit and 60% in the bar-tailed godwit.

A similarity plot, or dendogram, based on the relative amounts of β-hydroxy fatty acids and 1,2-diol with different chain lengths in the transesterified waxes, revealed that species within families resemble each other closest (fig. B3) and that species with shared ecologies (freshwater, subarctic or saltwater, high arctic environments) did not resemble each other much with respect to the preen wax composition.

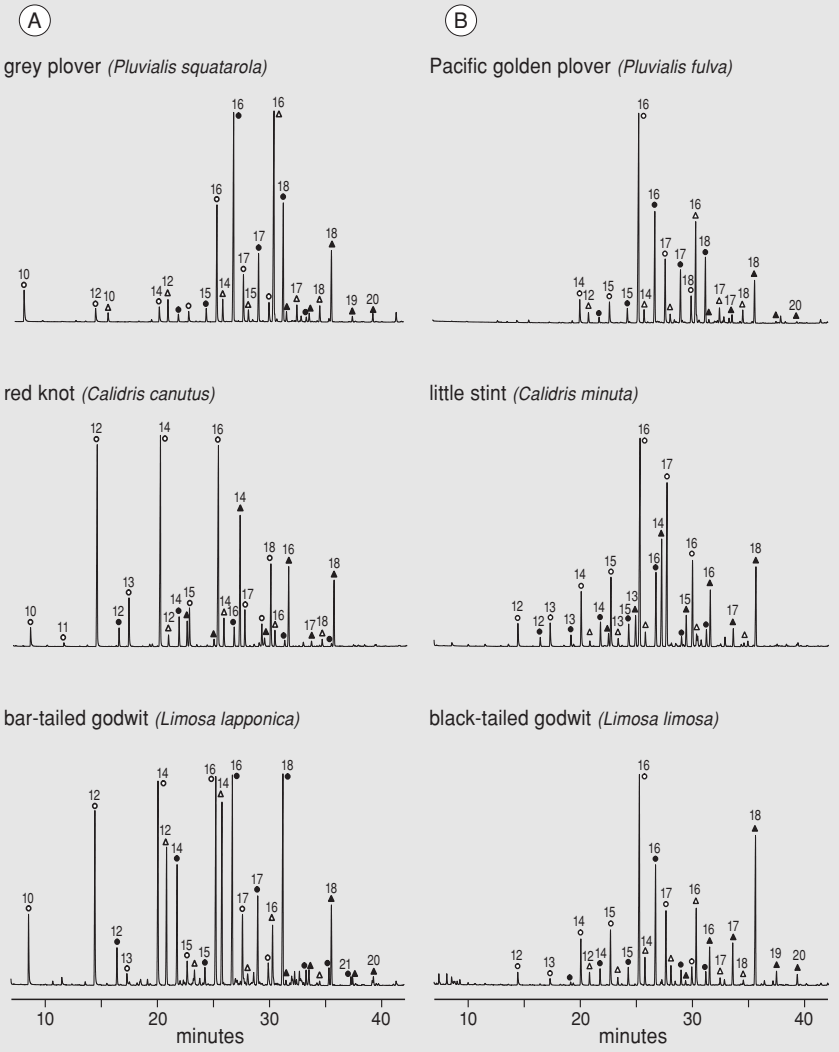

Figure B2 Gas chromatograms of the transesterified diester preen waxes of six shorebirds. The saltwater/High Arctic species are depicted in (A), the fresh water/subarctic species in (B). Open circles represent straight chain fatty acids, closed circles straight chain alcohols, open traingles ß-hydroxy fatty acids and closed traingles 1,2-diols. The numbers next to the symbols refer to the carbon chain length of the given compound.

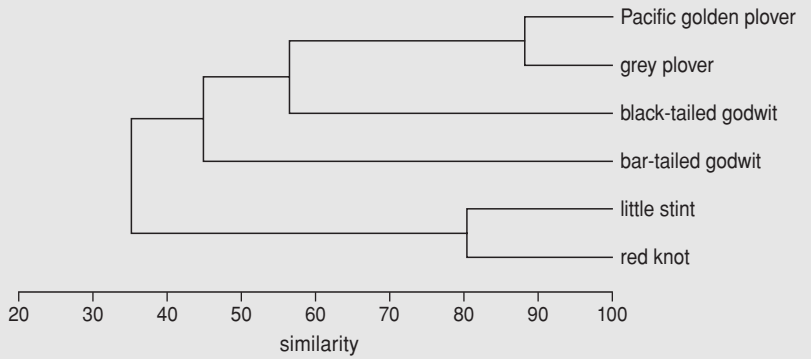

Figure B3 Dendograms based on Bray-Curtis similarity coefficients of species specific diester preen wax compounds of six shorebirds. The analysis is performed on the presence or absence of 1,2 diols and ß-hydroxy fatty acids with different carbon chain lengths.

Discussion

Although the investigated shorebird species show a large overlap in the range in carbon chain lengths of the secreted diester waxes, there is a lot of inter-specific variation in the chemical composition of the waxes. The majority of diester molecules of sandpipers was based on 1,2-diols esterified with fatty acids, whereas in the plovers diesters were mainly based on β-hydroxy fatty acids. This pattern suggests that the similarity in preen wax composition is largest between species within a family (fig. B3) and that the ecology of species plays a smaller role. The similarity between preen wax composition of the godwits are, however, not completely separated from those of the plovers. To our knowledge, this is the first time that the presence of intact diesters based on β-hydroxy fatty acids in avian preen gland secretions are shown. The presence of β-hydroxy fatty acids after transesterification of preen wax of mallards (*Anas platyrhynchos*; Kolattukudy *et al.* 1987) and wood pigeon (*Columba palumbus*, Jacob & Grimmer 1975) did, however already suggest that diesters based on β-hydroxy fatty acids occurred in preen gland secretions of some bird species.

Species-specific preen wax mixtures are probably the result of evolution by natural selection resulting in wax compositions that are best adapted to the demands of the environment of an individual (although selection can be constrained, e.g. Arnold 1992). Natural selection takes place on the secreted

Table B2 The relative percentage of the total of 1,2-diols and ß-hydroxy fatty acids in the transesterified diester preen wax of six shorebird species. The sum of the surfaces of each peak for each species were set to 100. The data for black-tailed godwit are of the individual of the *islandica* subspecies.

	red knot	little stint	black-tailed godwit	bar-tailed godwit	grey plover	Pacific golden plover
ß-OH-FA						
C_9	0	0	0	0	0	1
C_{10}	0	0	0	0	0	0
C_{11}	0	0	0	0	0	0
C_{12}	3	2	4	27	6	5
C_{13}	1	2	2	3	0	0
C_{14}	8	4	7	35	5	6
C_{15}	0	1	5	3	3	4
C_{16}	4	3	19	11	55	46
C_{17}	0	1	2	1	4	6
$C1_8$	2	1	1	1	3	5
1,2-diols						
C_{12}	7	4	0	1	0	0
C_{13}	2	9	0	0	0	0
C_{14}	35	29	1	1	0	0
C_{15}	3	8	1	0	0	0
C_{16}	19	13	9	1	2	2
C_{17}	1	4	9	3	2	4
C_{18}	15	18	35	12	14	17
C_{19}	0	0	3	1	1	1
C_{20}	0	1	2	1	2	1
C_{21}	0	0	0	0	0	0

mixture of intact diester waxes exposed to the environment. A comparative approach to examine the role of ecology such as this might increase our understanding of the selection pressures that have lead to the evolution of preen wax mixtures. The number of species is admittedly rather limited in the comparison presented here. Nevertheless, we were able to show that preen wax composition of closely related species resemble each other the most, suggesting that preen wax composition is evolutionary conservative. Because 1,2-diols and β-hydroxy fatty acids are never exposed to the environment (i.e. they are esterified with fatty acids and alcohols before secretion), it is perhaps not surprising that the chemical composition of species with shared ecologies resembled each other less than related species. For a

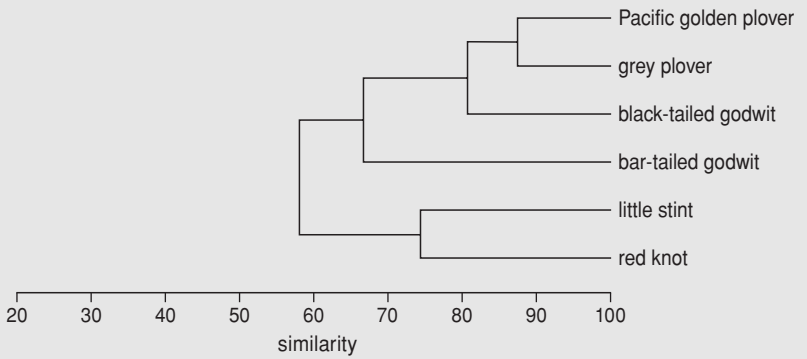

Figure B4 Dendograms based on Bray-Curtis similarity coefficients of species specific diester preen wax compounds of six shorebirds, based on corrected relative quantities of diester molecules with different carbon chain lengths.

proper analysis that would be able to distinguish between current selective pressures (environment, ecological niche) and selection over a longer time scale (phylogeny), we need detailed quantitative knowledge of the chemical composition of the intact diester waxes. These quantitative data are currently not available.

It is possible, nevertheless, to perform a crude analysis based on chain length of the intact waxes only, without paying attention to the chemical composition of the wax secretions. Such an analysis could yield insights into the natural selection on the chain length, which correlates with physical aspects such as viscosity, of the preen wax. The resulting dendogram of this similarity analysis (fig. B4) is similar to the one that includes information of the chemical composition (fig. B4), but the overall similarities between species are higher. Inclusion of data on the chemical composition thus yields a better distinction between species but does not change the overall pattern. This suggests that the largest variation in preen wax composition is the result of variation in the 1,2-diols and β-hydroxy fatty acids, and less in the straight-chain alcohols and fatty acids. That both the similarity analysis of the transesterified diesters and the crude analysis with chain lengths only yields similar results also suggests that the current diester preen wax mixtures have been shaped at the nodes of speciation and that selection pressures between related species with contrasting ecological traits are not strikingly different.

CHAPTER 3

Parental role division predicts avian preen wax cycles: a comparative study of sandpipers

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Submitted

ABSTRACT

Prior studies have shown that avian preen wax composition shifts from monoesters during most of the year to diesters during the breeding season for some classes of sandpipers (Scolopacidae). The production of diester waxes appears to reduce the olfactory cues available for mammalian predators to find nests. To investigate more thoroughly how the act of incubating might influence wax secretion, we examined seven sandpiper species with different incubation patterns, including species where both sexes incubate, only males incubate, or where only females incubate. During the breeding period, diester preen wax was secreted almost exclusively by the incubating sex in species with uniparental incubation, and by both sexes in species with biparental incubation. These findings suggest that diester preen waxes have a function that is directly related to the act of incubation. Unexpectedly, however, in curlew sandpipers and buff-breasted sandpipers – species with female-only incubation – some males secreted predominantly, or a small proportion, diester preen waxes during the breeding period. The presence of diesters in the males of these species suggest that some males may still incubate eggs, that these waxes may be a remnant from their evolutionary past when both sexes incubated and that selection pressures differed among species, or that males need to be olfactory cryptic because they are involved in the making of nest scrapes that will later be used by the females. The seasonal pattern of preen wax composition was also studied in five male and five female ruffs and of two female-mimicking males, so-called faeders, held in captivity. The captive female ruffs also changed preen wax composition to diesters in the spring despite the fact that no females actually laid eggs or were incubating. This suggests that circannual rhythms rather than actual incubation behaviour may trigger the shift to diester waxes. All captive male ruffs, including the two faeders, continued to secrete monoesters, confirming the idea that only the incubating sex secretes diesters.

Introduction

Ground-nesting bird species, and sandpipers in particular, show changing chemical composition of preen gland secretions throughout their annual cycle (Jacob *et al.* 1979, Kolattukudy *et al.* 1987, Piersma *et al.* 1999, chapter 2 and 4). Preen waxes are secreted by the uropygial gland ("preen gland"), and are applied onto the feathers by the bill during daily maintenance activities. Waxes probably help keep the plumage waterproof, reduce feather abrasion (Jacob & Ziswiler 1982) and repel feather-degrading mites and bacteria (Moyer *et al.* 2003, Shawkey *et al.* 2003, chapter 5). Changes in preen wax composition between seasons, however, suggest that waxes serve different functions during different periods within an annual cycle.

The most striking shift in preen wax composition of sandpipers occurs just before the start of the breeding season, when, within a few weeks, preen gland secretions consisting of only monoester waxes are replaced with waxes composed of only diesters (Piersma *et al.* 1999, chapter 2, Sinninghe Damsté *et al.* 2000). This chemical shift is presumably endogenously triggered (chapter 4). The secretion of diester preen waxes in sandpipers shows a clear temporal correlation with the breeding period (chapter 2). Experimental evidence shows that the less volatile diester preen waxes are more difficult for olfactory-searching predators to detect, and their use may thus help prevent nest discovery in the wild (chapter 7). An earlier hypothesis, that the shift to diester preen waxes might serve as an avian cosmetic (Piersma *et al.* 1999), has been rejected by Reneerkens & Korsten (chapter 5). Since diester waxes are not produced year-round, we infer that this potential advantage during incubation is outweighed by a different balance between costs and benefits at other times of the year. For example, it is not known if the physiological cost of producing these waxes differs, or if one of them is more effective in protecting feathers. To better understand the role incubation plays in the production of diester waxes, we investigated the presence of diester preen wax secretion during the breeding season among seven species of sandpipers specifically chosen because of their contrasting male and female incubation patterns.

Sandpipers are an ideal group of birds to investigate preen wax secretion because they show great diversity in breeding systems, ranging from polyandrous species with sole male parental care, biparental monogamous species with shared incubation and chick care, uniparental care at different nests by both sexes, and lekking species, in which males play no parental role beyond fertilisation (Pitelka *et al.* 1974, Piersma *et al.* 1996). This variation within a group of closely related species can be used in a comparative manner to investigate functional aspects of physiological traits related to the period of reproduction. For example, across

sandpiper species, the adrenocortical stress response during the breeding season in individuals that are most responsible for parental care are lower than those of individuals that are less responsible for parental care (O'Reilly & Wingfield 2001).

Methods

Breeding system of study species

The incidence of diester preen wax secretion was compared between males and females in sandpiper species with different mating systems. Species with biparental incubation included the red knot (*Calidris canutus*) and western sandpiper (*Calidris mauri*). Red knots are monogamous, and equally share incubation duties in shifts of 15-20 hours (Tulp *et al.* 1998); females usually depart soon after hatching leaving the males to care for the brood (Whitfield & Brade 1991, Harrington 2001). Western sandpipers are also monogamous and both parents incubate, but unlike red knots, males spend an increasing proportion of time incubating as the hatching date approaches (Erckmann 1981). After hatch, males also usually remain with the young longer than females (Holmes 1971). We studied five species with uniparental care. In Temminck's stint (*Calidris temminckii*), both sexes incubate and rear young, but do so independently (i.e., uniparental care with different clutches and broods). First clutches are incubated by the female's first mate, and the second clutch is incubated by the females themselves (Hildén 1975, Breiehagen 1988). In curlew sandpipers (*Calidris ferruginea*), pair bonds form, but incubation and parental care is solely by females (Holmes & Pitelka 1964, Tomkovich 1988), though males make scrapes which might form the future nest used by the female (Holmes & Pitelka 1964, Pitelka *et al.* 1974). In lekking buff-breasted sandpipers (*Tryngites subruficollis*) and ruffs (*Philomachus pugnax*) only females incubate and tend chicks (Lanctot & Laredo 1994; van Rhijn 1991) and males are not known to be involved in nest construction. Finally, in red phalaropes (*Phalaropus fulicarius*) sex roles are reversed and males provide all care during incubation and chick guarding (Tracy *et al.* 2002).

Sampling birds in the field and in captivity

All investigated sandpiper species breed either in the Arctic or sub-Arctic tundra, except for ruffs which also breed in temperate climate zones (Piersma *et al.* 1996). Birds were typically caught during territory establishment and incubation (i.e., May through early July), although ruffs were caught during migration *en route* to breeding areas located farther north.

We captured birds during staging and pre-nesting mainly by using wind-assisted clap nets, and during incubation with small spring-triggered bow nets placed over nests. Individuals that do not incubate (e.g., male buff-breasted sandpipers, male curlew sandpipers, and female red phalaropes) were captured prior to the start of incubation. This was necessary since these individuals typically leave the breeding grounds soon after females are fertilised (van de Kam *et al.* 2004) and would be impossible to capture otherwise. Capturing birds at this time should not bias the detection of diesters since previous studies indicate that most (ca. 80%) sandpipers that do incubate, to have completed the shift in to diester preen waxes during the courtship period (chapter 2). Birds guarding chicks were excluded from the analysis, as they are known to secrete monoester preen waxes only (chapter 2).

To provide a detailed profile of seasonal change and its potential direct relationship with incubation behaviour, we sampled five male and five female captive ruffs on a weekly basis between 4 April and 11 July 2001. Most of these females laid eggs, but these were removed immediately and the females did not incubate nests. Two experimental males died prior to the end of the season, reducing the number of male samples for the latter part of the season. In addition to these ten captive birds, we collected preen wax samples from two captive "faeder" ruffs; rare males that permanently mimic females as part of their mating strategy (Jukema & Piersma 2006). The sexually active faeders were sampled on 5 June 2006, at the height of the breeding season.

Seasonal shifts in preen wax composition have not previously been described for buff-breasted sandpipers. Therefore, in addition to samples from breeding grounds in Alaska, preen wax samples were also collected from a wintering location in Brazil, in December 2001. More details on the locations of the study sites are given in table 3.1.

Sexing methods

On all individuals, we measured bill length, wing length (maximum chord, stretched and flattened), tarsus, and total head (head and bill) to the nearest mm, and body mass to the nearest gram. We used these measurements either directly or indirectly (e.g., discriminant function analysis) to separate the sexes of ruffs (Jukema & Piersma 2006), buff-breasted sandpipers (R. Lanctot, unpubl. data), curlew sandpipers (Prater *et al.* 1977), and western sandpipers (Page & Fearis 1971). Exceptions to this included red phalaropes that can be sexed reliably on the basis of their alternate plumage (Prater *et al.* 1977), and red knot and Temminck's stint, which were sexed using molecular techniques (modified protocol after Griffiths *et al.* 1998, Baker *et al.* 1999). Additionally, buff-breasted sandpipers were caught in leks and behaviour of the individuals before being

caught confirmed sex assignment. We also used molecular methods to confirm the sexes of 13 of the 46 curlew sandpipers; all assignments were consistent with those made in the field by size and plumage only.

Preen wax collection and analysis

Preen wax was sampled by carefully making a smear of the papilla of the preen gland with a cotton bud. The cotton buds with collected waxes were wrapped in aluminium foil to avoid contamination and stored at room temperatures or kept in refrigeration before shipment to the laboratory of Royal Netherlands Institute for Sea Research for chemical analysis. The composition of the preen wax secretions was determined on the basis of the characteristic gas chromatogram patterns. These patterns were verified by gas chromatography/mass spectrometry of complete and hydrolysed waxes (cf. Dekker *et al.* 2000, Sinninghe Damsté *et al.* 2000) secreted by both sexes of each species. The fraction of diesters in the secretions was estimated by measuring the surface of the typical diester peaks in the gas chromatograms of diesters divided by the sum of the surface of all peaks (monoesters and diesters).

In cases when, as described for red knots, two distinct monoester wax mixtures were identified - 'monoesters A' and 'monoesters B'- (Sinninghe Damsté *et al.* 2000, chapter 4), we determined the relative abundance in the secretions by measuring the surface of the highest peaks of the two monoester mixtures in the gas chromatograms only (cf. chapter 4). In contrast to peaks of diester waxes, the peaks in the gas chromatogram of monoesters A and B overlap and are usually difficult to discriminate visually.

Statistical analysis

We used a generalised linear mixed model with the fraction of diesters in individual preen wax samples as the response variable, 'species' as a random variable and parental care system (both sexes incubate, whether at the same or different nests; and male-only or female-only incubation) and sex as fixed variables. We used a logit link function in view of the binomial distribution of the data. Wald tests were used to test for the significance of fixed effects at the level of 95% and all two-way interaction terms were tested. The samples of wintering buff-breasted sandpipers were excluded from this analysis. Within the group of calidridine sandpipers and within breeding systems, the species in this study are not closely related (Borowik & McLennan 1999). Therefore, we did not lump the species within a breeding system and did not use any phylogenetic correction in our statistical tests.

Table 3.1 Percentage of individuals that secreted diester waxes for seven sandpiper species with different parental care systems. Sample size for each sex and the average fraction of the preen wax sample of all individuals that was composed of diesters is listed in parentheses.

Species	Role division incubation	Site[a]	Sample Period	Life cycle stage	Diester secretion[b] male		Diester secretion[b] female	
red knot (*Calidris canutus*)	biparental	A,B,E	1 June- 14 July	pre-nesting and incubation	100	(24, 0.94)	100	(16, 1.00)
western sandpiper (*Calidris mauri*)	biparental	C	7 June- 6 July	incubation	100	(17, 1.00)	100	(18, 0.96)
Temminck's stint (*Calidris temminckii*)	uniparental by both parents	D	2 June -7 July	pre-nesting and incubation	100	(27, 1.00)	100	(29, 1.00)
curlew sandpiper (*Calidris ferruginea*)	female-only	E	7 June – 16 July	pre-nesting and incubation	46.2	(13, 0.43)	97.0	(33, 0.94)
ruff (*Philomachus pugnax*)	female-only	F	14 March-17 May	spring migration	0	(47, 0.00)	51.6	(31, 0.28)
buff-breasted sandpiper (*Tryngites subruficollis*)	female-only	G	5-7 June	pre-nesting	85.7	(14, 0.07)	100	(5, 1.00)
		H	12-23 December	wintering	0	(15, 0.00)	0	(14, 0.00)
red phalarope (*Phalaropus fulicarius*)	male-only	E	24 June -21 July	pre-nesting and incubation	85.7	(21, 0.70)	0	(12, 0.00)

[a] Letter refers to the following study sites: A = Alert, Ellesmere Island, Canada, B = Zackenberg, northeast Greenland, C = Kanagayak, Yukon-Kuskowin Delta, western Alaska, D = Enontekiö and Oulu, Finland, E = various locations in Siberia, Russia (Medusa bay, Taimyr peninsula and Chukotka, east Siberia) F = Friesland, The Netherlands, G = Prudhoe Bay, Alaska, H = Estação Ecológica do Taim, Rio Grande, Brazil.

[b] See text for the method used to estimate the fraction of diesters in the preen gland secretions of individual birds.

Results

Six of the seven sandpiper species produced both mono- and diesters in their preen wax (table 3.1). The one exception to this pattern was Temminck's sandpipers where both males and females secreted only pure diester preen waxes (table 3.1). At wintering sites, both male and female buff-breasted sandpipers secreted pure monoester waxes, whereas diester preen waxes were only secreted during the breeding season (table 3.1). In all species investigated, the total carbon number distribution of the secreted diester waxes ranged between C_{34} and C_{50}, but in a few species (red knot, ruff, curlew sandpiper) small amounts of C_{30}-C_{32} diesters were also found and Temminck's stints also secreted C_{52} diesters. The majority of the diesters comprise 1,2-diols esterified with straight chain fatty acids at both positions, but (part of) the shorter chained diesters comprise β-hydroxy fatty acids esterified with a fatty acid at one and an alcohol at the other position (C_{32}-C_{39} diesters in curlew sandpipers, C_{34}-C_{37} diesters in western sandpipers, C_{32}-C_{40} diesters in ruffs and C_{35}-C_{43} diesters in red phalarope). Temminck's stints secreted diesters based on 1,2-diols only.

The secretion of diester preen waxes during the breeding season varied with the sex of the birds within a given breeding system (generalised linear mixed model; Wald statistic = 7.18, df = 2, P= 0.028). Diester secretion occurred equally often and always in both males and females of species where both sexes incubate (red knot, western sandpiper and Temminck's stint; table 3.1). In the one investigated species with male-only incubation, the red phalarope, only males secreted diester preen waxes although three of the 21 incubating males did not (table 3.1).

In species with female-only incubation (ruff, curlew sandpiper and buff-breasted sandpiper), nearly all females secreted diester preen wax during the breeding season, although at varying concentrations. In buff-breasted sandpiper, all females switched entirely to (diol-based) diesters during the breeding season. Male ruffs never produced diesters (table 3.1). Perhaps unexpectedly, diesters occurred in some male curlew sandpipers and buff-breasted sandpipers (table 3.1). Six of 13 curlew sandpiper males secreted diesters during the breeding season (average percentage in all males was 43%). Both male and female buff-breasted sandpipers secreted the same monoester preen wax during winter. Male buff-breasted sandpipers continued to secrete mainly monoesters during the breeding season, but of a different composition and with a small percentage of diesters (fig. 3.1, table 3.1). The diesters produced by male buff-breasted sandpipers had carbon chain lengths of C_{36}- C_{50} with an even over odd dominance, as in red knots (Sinninghe Damsté *et al.* 2000). Females also secreted shorter diesters with carbon chain lengths between C_{24}- C_{50}.

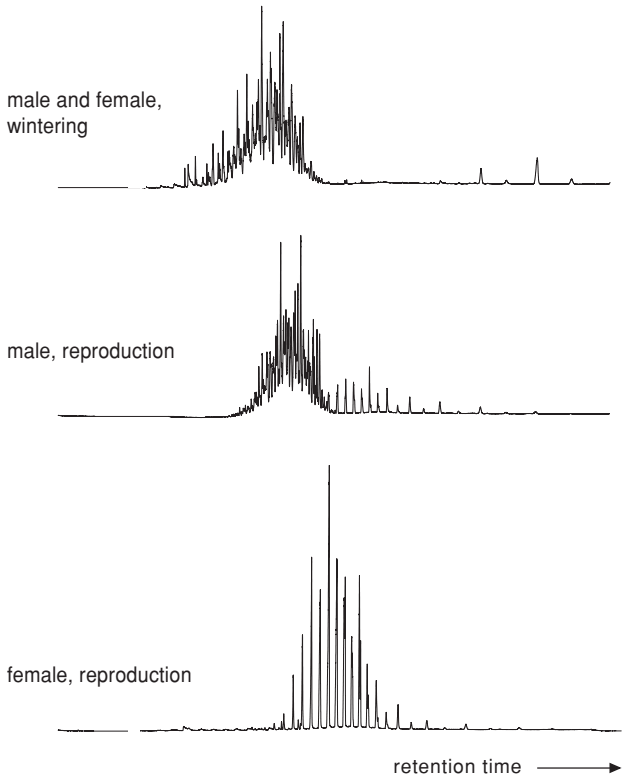


Figure 3.1 Typical gas chromatograms of female and male buff-breasted sandpiper in winter (top) and in the Arctic during the pre-breeding period of males (middle) and females (bottom). Note that the typical diester peaks in the gas chromatogram of females in the reproductive period are also visible (right) in the predominantly monoester based gas chromatograms of males during reproduction.

Shifts from mono- to diester preen waxes occurred prior to the actual start of incubation, during the period of courtship and mate choice on the breeding grounds in all species investigated here (cf. chapter 2). In ruffs, several females already secreted diesters during migratory refuelling on a stop-over site in The Netherlands (table 3.1, fig. 3.2), but none of the males did.

All five captive female ruffs that were repeatedly sampled exhibited a change in preen wax composition from monoesters to diesters at the beginning of the breeding season, and then reverted to monoesters following breeding (fig. 3.2). Like the free-living ruffs, the monoester preen wax in the captive females changed from one distinct monoester mixture in winter (monoesters A) to another

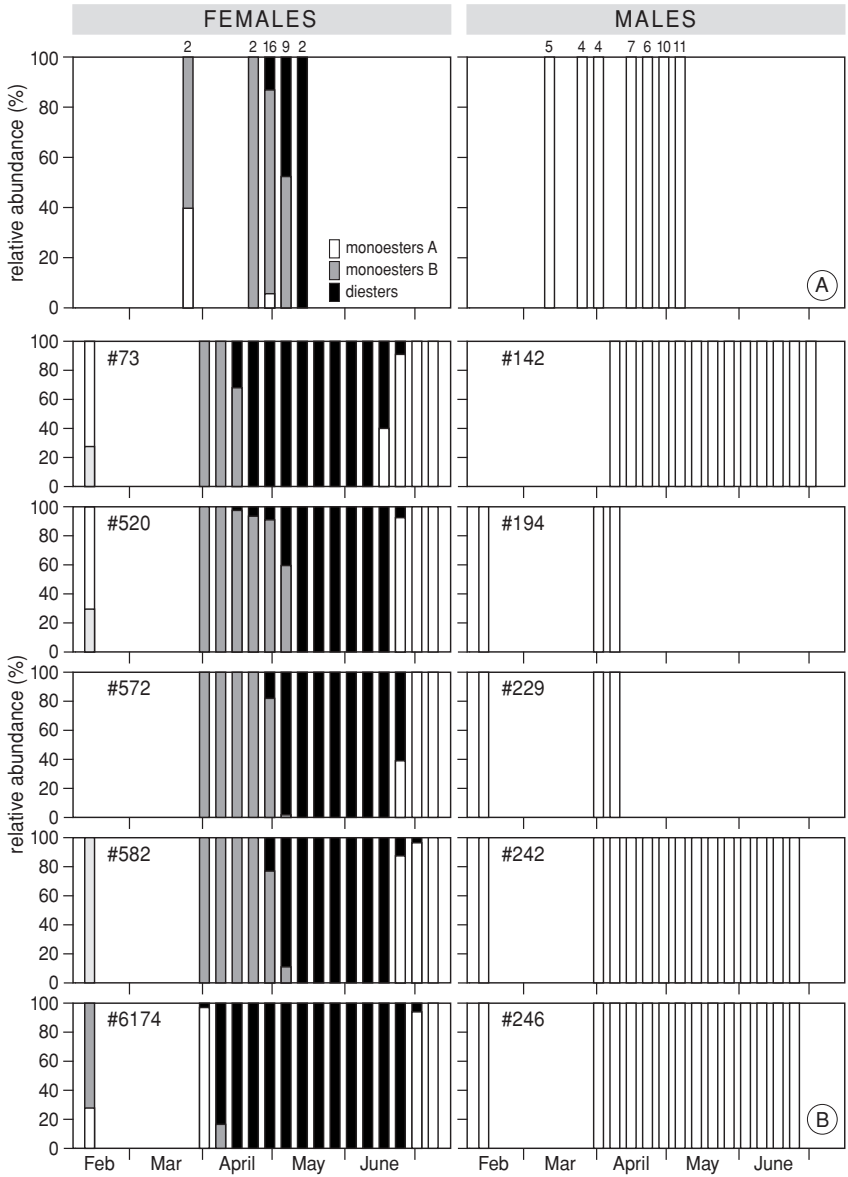


Figure 3.2 Seasonal changes in preen wax composition of free-living (A) and captive (B) ruffs in spring. Average preen wax composition per week is shown with monoester A in white stripe, monoesters B shown in grey, and diesters in black. Numbers in parentheses in (A) indicate sample sizes in each week. Each block in B represents individual birds, which are denoted by their ring numbers (#).

(monoesters B; chapter 4; fig. 3.2) before wax composition shifted to diesters. In contrast, male ruffs in both the free-living and captive situations secreted only monoester A preen waxes throughout the spring and summer, including the two captive faeders (male ruffs that mimic females in plumage; Jukema & Piersma 2006) in early June, at the height of their breeding season.

Discussion

We showed that in seven sandpiper species with varying parental care systems, diester preen wax secretion during the pre-incubation period of post-migratory arrival, courtship and egg-laying and during incubation (cf. chapter 2), occurred primarily in the incubating sex or sexes. In species where both sexes incubate, diester preen waxes were secreted by both males and females during incubation. In species in which only one of the two sexes incubates, diester preen wax secretion occurred only (or mainly) in the incubating sex. The portion of diesters in preen waxes of the incubating sex was only less than 90% for ruffs (table 3.1), because individuals from this species were caught during spring migration. In the captive population of ruffs all females shifted from monoesters to pure diester preen waxes shortly before the start of the breeding season in wild ruffs (fig. 3.2). Although a single female curlew sandpiper and three male red phalaropes did not secrete (pure) diesters during incubation, the overall pattern strongly suggests that diester preen waxes are most important for sandpipers that incubate. That only incubating adults secrete diester preen waxes makes sense given that Reneerkens *et al.* (chapter 7) experimentally showed that the less volatile diester preen waxes were more difficult to detect by a sniffer dog than were monoester waxes. The secretion of diester preen waxes appears to make incubating birds more 'olfactory cryptic' and thus less detectable to mammalian predators.

If shifts to diester preen waxes only occur in individuals of the sex that usually incubates, why then do birds secrete diesters prior to incubation, such as during courtship and egg laying (chapter 2) and spring migration in red knots (Piersma *et al.* 1999) and ruffs (fig. 3.2)? Like the captive ruffs in this study, red knots in captivity switched from monoester to diester preen waxes during the time their free-living conspecifics would also do this, even though they did not actually incubate. Preen wax shifts from mono- to diesters in red knots appear to be under endogenous control (chapter 4), which enables feathers to be coated with diesters in time for the onset of incubation and which may explain why diester preen waxes are secreted and presumably accumulate on feathers for several weeks before the potential onset of incubation in most individuals. We suggest that this endogenous control causes birds to secrete diester preen waxes already before the start of incubation.

Diester preen waxes unlikely play a role as a visual quality signal or 'avian make-up' (chapter 5) during the pre-incubation period. The increased difficulty for predators to smell diester preen waxes compared with monoesters as shown by Reneerkens *et al.* (chapter 7) may, however, already be important before the actual start of incubation. Arctic sandpipers create nest cups by scraping their breast on the tundra and may thereby unintentionally transfer preen waxes from their feathers into the nest cups. These potential olfactory traces could make the nest less liable to detection by ground predators using olfactory cues during the egg-laying period. This may have particular selective consequences in the early High Arctic breeding season when in some years snow cover could conceivably narrow the search area that predators would have to cover to find sandpiper nests.

The importance of secretion of less volatile diester waxes during nest building may also explain why the dichotomy in diester preen wax secretion between sexes is not absolute in two of the three species with female-only incubation. Six of the 13 male curlew sandpipers secreted diesters and the preen wax of 12 of the 14 male buff-breasted sandpipers also contained diesters during the pre-nesting period, although only very small amounts (an average of 7% compared to the 43% in male curlew sandpipers; table 3.1). These differences in diester secretions in males of sandpipers with female-only incubation could be explained with species differences in the contribution of males in nest construction. Even though male curlew sandpipers are thought not to take part in incubation, they do assist with nest scraping (Holmes & Pitelka 1964). This has, as far as we know, never been described for males of ruff and buff-breasted sandpiper. It is possible that nest scraping behaviour has selectively favoured evolution of seasonal preen wax shifts in male curlew sandpipers. This hypothesis, however, can not explain why only part of the male curlew sandpipers shifted to diesters, except when only some males make nest scrapes. Neither can it explain why most male buff-breasted sandpipers secrete small amounts of diesters during the pre-incubation period. The latter might indicate more involvement of males in the nest building process or another aspect of buff-breasted sandpiper biology that is not presently appreciated, but then the question remains why they do not secrete pure diesters.

An alternative explanation why male buff-breasted sandpipers and curlew sandpipers might secrete diester waxes, although only in small amounts in the former species, is that the diester secretion is a remnant of an evolutionary past when both males and females shared incubation. Based on phylogenetic patterns of parental care, Borowik & McLennan (1999) suggested that biparental incubation is ancestral in calidridine sandpipers and that curlew sandpipers lost male care, as did buff-breasted sandpipers and ruffs. Male curlew sandpipers sometimes develop (incomplete) brood patches (Tomkovich 1988, Tomkovich &

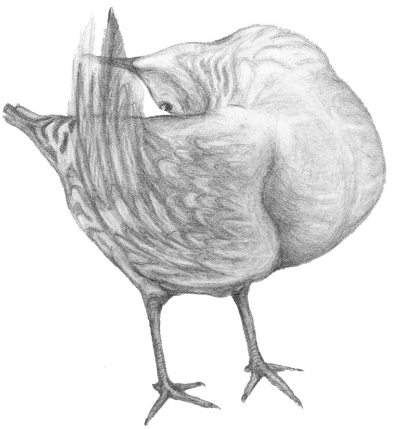
Soloviev 2006), which is consistent with this hypothesis. Considering this scenario, the (partial) shifts to diester preen waxes in male curlew sandpipers and male buff-breasted sandpipers might be a remnant of their past, a time when both sexes did incubate. Ruff would be the only species of the three with female-only incubation in which males have completely lost the ability of producing diester preen waxes. A recent phylogenetic reconstruction of the sandpiper family shows that the Ruff divergence is very ancient and happened at about the same time as that of curlew sandpipers (A.J. Baker unpubl.). The phylogenetic reconstruction shows that the divergence of buff-breasted sandpipers is rather old too. Therefore, we suggest that the recent diester preen wax secretion by male buff-breasted sandpipers and male curlew sandpipers might indicate that the losses of male incubation in these species have occurred more recently, or else that these species have been subjected to different selection pressures.

Faeders, male ruffs that mimic females in plumage and "sneak" copulations at leks, secrete monoesters like other males. Faeders have been proposed to represent the ancestral male type of ruffs (Jukema & Piersma 2004) which presumably participated in incubation (van Rhijn 1985). Give the strong correlation between incubation and diester preen wax secretion, the lack of diester preen waxes suggests that faeders are unlikely to participate in any incubation duties at the present time. Consistent with this, behavioural observations of faeders in captive breeding situations have shown no indication that faeders participate in nesting or incubation (DBL, S. McCrae, TP & JJ, unpubl. obs.). Faeders, and 'normal' male ruffs, might have been subjected to strong selection pressures to eliminate diester production in their evolutionary past and consequently have lost this physiological characteristic related to incubation.

The function of the shift from monoesters A to monoesters B, which occurs in female ruffs (fig. 3.2) and in both sexes of red knot (chapter 4), remains unclear. The fact that only female ruffs shift into producing monoesters B suggests that this wax is a discrete transient mixture that is secreted when biosynthesis of monoesters A is changing to diesters. However, because many of the fatty acids that compose monoesters B in red knots have branched (methyl-substituted) carbon chains, whereas those that compose diester preen waxes are unbranched (JR, JSSD, WICR & TP unpubl. data), we believe that different types of fatty acids have to be synthesised for each preen wax mixture. Male buff-breasted sandpipers produce different monoesters in winter and summer, suggesting that they are producing monoesters A and B, as opposed to male ruffs, that only produce monoester A. The occurrence of a monoester A (during winter) and monoester B (spring) type is now known to occur in many more sandpiper species (JR, JSSD, WICR & TP unpubl. data).

In summary, this comparative study on sandpipers revealed that seasonal shifts in preen wax composition from monoester to diester preen waxes are largely restricted to incubating birds, but also occur (facultatively or in small concentrations) in some males of species that presumably have lost paternal care. In these males increasing olfactory crypsis by seasonal preen wax shifts may also be involved because they make nest scrapes, but this remains to be investigated. While we suggest that diester preen waxes are useful during nest construction and incubation for birds to become more olfactory cryptic for mammalian predators, we do not yet understand what their drawbacks are for use under other conditions. Possibilities include higher production costs and/or lower effectiveness with respect to the alternative functions of preen waxes described earlier. We suggest that future studies should experimentally address the premises of differences between the monoester and diester preen waxes relative to cost in syntheses and efficiency in protecting feathers.

Acknowledgements
We like to thank Kim Mathot, Amanda Niehaus, Amanda Pomeroy, Dana Seaman, Antti Rönkä, Kari Koivula, Veli-Matti Pakanen and many Frisian bird catchers ('wilsterflappers') for their help with bird catching and collection of preen wax samples. The manuscript benefited much from a fruitful discussion with Pieternella Luttikhuizen. The work was financially supported by the Netherlands Organisation for Scientific Research (NWO) through ALW grant 810.34.003 and PIONIER grants to TP and JSSD, by CNPq – a Brazilian Government entity for scientific and technological development – to JBA, by the National Science and Research Council of Canada (NSERC) to DBL and the US Fish and Wildlife Service to RBL.



CHAPTER 4

Expression of annual cycles in preen wax composition in red knots: constraints on the changing phenotype

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Journal of Experimental Zoology Part A, in press

ABSTRACT

Birds living in seasonal environments change physiology and behavior in correspondence to temporally changing environmental supplies, demands and opportunities. We recently discovered that the chemical composition of uropygial gland secretions of sandpipers (Scolopacidae, order Charadriformes) changes abruptly at the start of the breeding season from mixtures of monoesters to diesters. Diester preen waxes have been shown to be functional during incubation but probably entail certain net costs in other periods; this may explain the seasonal changes in preen wax composition. A proper temporal match between the expression of diester preen waxes and incubation requires a flexible organization of the trait. Here we analyze the possible degrees of flexibility with reference to the functionality of better understood molt and body mass cycles of free-living and captive red knots (*Calidris canutus*). The relative flexibility of seasonal cycles in preen wax composition was examined by two experimental perturbations: (1) giving birds restricted access to food and (2) monitoring them long-term under a constant photoperiodic regime. Just as molt and mass cycles, the seasonal rhythm of diester secretion appeared under endogenous control: most birds placed in a constant photoperiod still maintained seasonally changing preen waxes. Diester preen wax secretion was synchronized with the peak in body mass in spring, but became less well expressed under constant photoperiodic conditions and when food availability was limited. Perhaps surprisingly, we found that wax type cannot change instantaneously, but that changing the type of wax is under similar organizational time constraints as the replacement of feathers.

Introduction

Animals living in seasonal environments exhibit phenotypic flexibility in physiology and behavior in an environmental context (e.g. Barta *et al.* 2006, Piersma, 2002a, Piersma & Drent 2003, Wingfield 2005). Phenotypic plasticity (West-Eberhard 1989, Stearns 1989) can occur at different organizational levels (between and within generations) and time scales (days, months, years; Piersma & Drent 2003). If time scales and the magnitude of phenotypic change are adaptive, the nature of phenotypic change can inform us about the functioning, and possible changes of function, of particular traits.

Here we will examine one such trait from this perspective. Preen waxes are secreted by the uropygial gland and smeared onto the plumage to protect feathers against adverse environmental conditions that cause wear or wetting (Elder 1954, Jacob & Ziswiler 1982) and to protect the plumage against ectoparasites (Moyer *et al.* 2003, Shawkey *et al.* 2003). During courtship and incubation the usual preen wax mixture of monoesters, secreted during the non-breeding season, is replaced by higher molecular weight diesters in female mallards (*Anas platyrhynchos*; Kolattukudy *et al.* 1987) and in many species of sandpiper (Scolopacidae; chapters 2 and 3). For red knots (*Calidris canutus*) there is evidence that diesters are less smelly than monoesters, a trait that is particularly important for birds that incubate a clutch in nests that are fully exposed to mammalian predators (chapter 7). The fact that diester preen waxes are not secreted year-round suggests that there are costs associated with diester production, – secretion and/or – application, that are outweighed by their benefits (a possible reduced chance of predation of eggs) only during incubation (chapter 3).

If diester preen waxes are costly to make and important for 'olfactory crypsis' during incubation, but not during other life cycle stages, and if preen wax composition can be changed instantaneously, diester preen waxes should not be secreted until the day incubation starts. When red knots skip a breeding season they should forego producing diester preen waxes; when they lose a clutch to a predator, preen wax composition should instantaneously change to monoesters.

Many seasonal changes in phenotype of migratory birds are endogenously controlled (Gwinner 1986, 1990). Endogenous regulation of phenotypic traits is adaptive when it takes a relatively long time to change from one state to another state. The phenotypic change should then be initiated before the changed phenotype is actually needed. Complete wing feather replacement in shorebirds such as red knots lasts 60-100 days (Boere 1976), and is endogenously controlled (Cadée *et al.* 1996, Piersma 2002b). A complete shift in preen wax composition from monoesters to diesters seems to happen at the level of a population within a few days (chapter 2). Red knots secrete three distinct preen wax types during an an-

nual cycle (Dekker *et al.* 2000, Sinninghe Damsté *et al.* 2000) all of which consist of esters, which are alcohols condensed to fatty acids. Because the chemical make-up of the three preen wax types is essentially similar, we expect that an instantaneous shift from one to another type of preen wax mixture is possible as long as the required fatty acids and alcohols are available.

However, if changing the production from monoester to diester preen waxes would entail considerable modifications of the biochemical or metabolic machinery, the changes would need to be pre-arranged in time. Just like molt and pre-migratory fuelling, the organization would then be more in need of some sort of endogenous control. In examining the organization of seasonal changes in preen wax we will focus on the shift from monoesters to diesters because studies of its function clearly predict these shifts to best occur instantaneously. We examined flexibility in shifts to diester preen wax secretion with reference to the better understood annual cycles in molt and pre-migratory fuelling in free-living and captive birds. To tease apart the expression of the different phenotypic traits, annual cycles were perturbed by either limiting the daily food resources or by placing birds in a constant photoperiodic regime. Under the assumption that a change from mono- to diester preen waxes at the start of incubation best occurs overnight, we predicted (1) that red knots in captivity would stop producing (costly) diester preen waxes (as they do not breed in captivity), and (2) birds in the treatment groups would first stop expressing diester preen waxes before 'giving up' molt and body mass cycles.

Methods

Birds

Next to the 159 preen wax samples analyzed by Reneerkens *et al.* (chapter 2), 47 additional samples of wild red knots were collected in several different months in the year at different locations worldwide, to get a complete overview of preen wax composition year-round. Based on plumage characteristics (Prater *et al.* 1977), we know that all birds in our analysis were in their second calendar year or older.

Twenty-seven red knots of the subspecies *islandica* were caught with mist-nets at night at high tide roosts in the Dutch Wadden Sea between October 1995 and August 2000. The birds were brought into captivity at NIOZ, Texel, within 100 km from their catch sites. The birds were maintained in five separate outdoor aviaries where they experienced the local light regime and ambient temperatures. Eleven birds in two of the aviaries were food restricted between March 6, 2002 and July 9, 2002. During this period the birds daily obtained *ad libitum*

trout pellets for 6 hrs (between 10 AM and 4 PM) only. A different group of fourteen red knots were caught in October and November 1994 (except for one bird that was caught in October 1995) and kept in outdoor aviaries until November 26, 1996, when they were placed in two separate indoor aviaries with a constant photoperiod of 12 hrs: 12 hrs light-dark (L: D) cycle. Air temperature was kept constant at 15ºC in the indoor facilities with occasional daily peaks of 25ºC in summer (June- September). All red knots were individually banded with a numbered metal ring.

All aviaries measured 2 m x 4 m with a height of approximately 2 m and contained a small area of 'mudflat' with continuously flowing seawater. The hard surface of the aviaries was kept wet with seawater also. A through flow freshwater basin was used by the birds to bath and drink (see Piersma & Ramenofsky 1998 for a photograph of such an aviary). Birds were fed protein-rich trout pellets that were always available *ad libitum*. All red knots were older than two years at the start of the experiment. Each aviary, indoor and outdoor, contained both male and female red knots, but females were in minority in each treatment group (sex ratio's of 10:5, 8:3, and 10:4 males: females in the control, the food restricted and the birds in a constant photoperiodic regime, respectively). Because male and female red knots have the same timing of spring migration and because both sexes incubate, no differences in annual cycles were expected.

At weekly intervals the birds were taken out of their aviaries. As the aviaries were cleaned and disinfected, body mass was measured at least an hour after they could last have fed. Intensity of contour feather molt (0: no molt, 1: little molt, 2: intermediate molt, and 3: intensive molt) and the amount of breeding plumage (from 1, complete winter plumage, to 7, complete breeding plumage) were scored. In our analyses we determined average daily mass gain by subtracting the body mass value during the preceding measurement from the measurement following the date at which daily mass gain was determined, divided by the number of days between these measurements. The end of the mass plateau in spring was determined by selecting the first date at which birds weighed more than their average during the experiment and lost at least 14 g within the next week. Preen wax from the uropygial gland was sampled by carefully massaging the uropygial gland with a cotton bud.

All captive birds were studied for 17 months, except the birds that were food restricted that were studied for six months between February and August 2002 and of which preen wax samples (253 in total) were collected between May 7 and July 22, 2002. The red knots in the outdoor aviaries were studied between February 5, 2002 and July 1, 2003. In the period between June and August 2002 body mass and molt was measured and preen wax sampled on a bi-weekly basis of this group of birds, as well as of the birds that were food restricted. In total

1210 preen wax samples were collected of the birds in outdoor aviaries that received food *ad libitum* throughout the experiment. The 14 birds in the invariable, indoor environment were studied from February 4, 2001 to July 4, 2002, but preen wax was only sampled for twelve months between March 6, 2001 and March 12, 2002 (616 samples in total). Red knot #485 (outdoor aviaries) died at May 24, 2003, six weeks before the end of the experiment, of unknown cause.

Preen wax collection and characterization

We used the procedures of molecular analysis of preen waxes by gas chromatography as described by Dekker *et al.* (2000). Briefly, preen wax was brought into solution with ethyl acetate (1 mg wax/ml ethyl acetate). The samples were injected into a gas chromatograph (Hewlett-Packard 6890 Series II, using a fused-silica capillary column) at 70 ºC, subsequently heated to 130 ºC at 20 ºC min^{-1} and then at 4 ºC min^{-1} to 320 ºC where the oven temperature was held at for 35 min.

To determine the relative abundance of the different wax mixtures in a sample, the highest peak in a pure mixture of monoesters A, monoesters B and diesters was selected. The peak of hexadecyl 2-methyloctanoate (Dekker *et al.* 2000) was indicative of monoesters A, the peak of octadecyl 2-hexanoate for monoesters B, and for the easily recognizable mixture of diesters the highest peak in the mixture, 1,2-tetradecanediyl 1-hexadecanoate 2-octadecanoate (Sinninghe Damsté *et al.* 2000), was used (fig. 4.1). Using the integration software, we estimated the relative surface area of each of the three selected peaks and used this as an estimation of the proportion of different waxes in the secretions.

Results

Mass and molt under natural photoperiods

Captive red knots subjected to natural photoperiodic conditions maintain annual cycles in molt and body mass (Piersma & Ramenofsky 1998), cycles that are comparable to those of free-living birds (Piersma *et al.* 1995). In May-early June they showed peaks in body mass coinciding with the natural period of northward migration to the Arctic breeding grounds (fig. 4.2). During their first two years in captivity, *islandica* red knots maintain a mass peak in winter (Piersma & Ramenofsky 1998), but these peaks then disappear (TP personal observations) as they did in the long-term captive birds studied here.

The contour feather molt in spring began before the increase in body mass (fig. 4.2). By the time the birds had obtained maximal fuel stores, at the end of June, they were in (almost) complete alternate plumage. When the birds had lost their fuel stores by fasting for several days, a molt towards a basic plumage

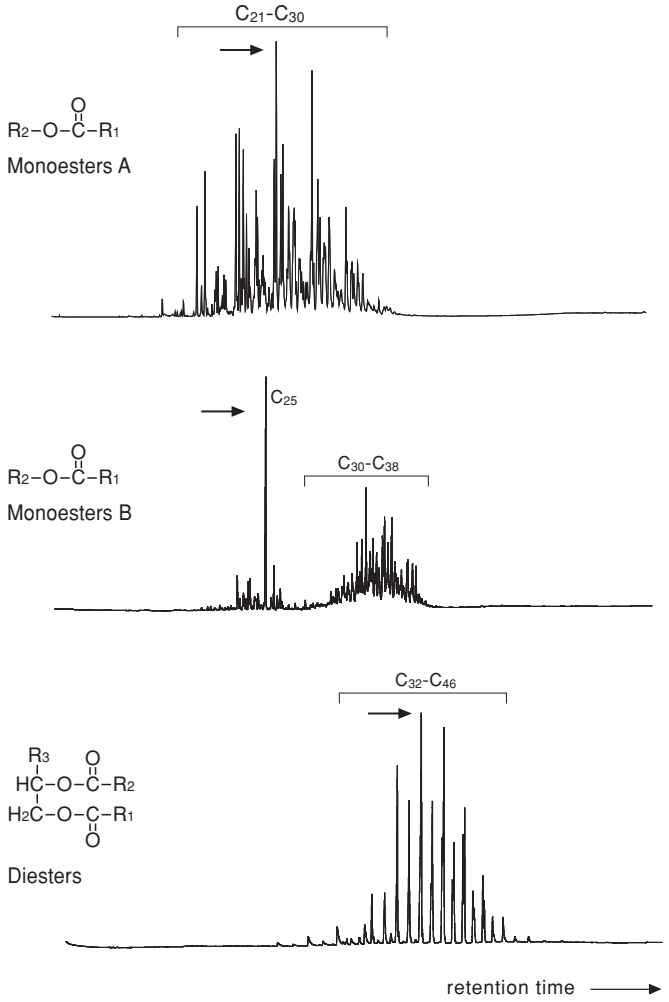


Figure 4.1 Gas chromatograms of pure monoesters A, monoesters B and diesters. Monoesters A are composed of only C_{21}-C_{30} monoester waxes composed of C_7-C_{16} 2-methyl and 2,6- and 2,8-, and 2,10-dimethyl fatty acids esterified with C_{11}-C_{22} straight-chain and methyl-branched alcohols. Monoesters B have a total carbon number distribution in the range C_{24}-C_{26} and C_{30}-C_{38} predominantly based on C_{17}-C_{19} alcohols. Diesters consist of C_{32}-C_{48} ester waxes predominantly comprising C_{12}-C_{16} alkane-1,2-diols esterified with C_8, C_{10}, and C_{12} fatty acids at one, and predominantly even-numbered carbon fatty acids at the other position. Arrows indicate the peaks typical for each preen wax type, that were used to estimate the proportion of each wax type in the sampled preen wax secretions, hexadecyl 2-methyloctanoate for monoesters A, octadecyl 2-hexanoate for monoesters B and 1,2-tetradecanediyl 1-hexadecanoate 2-octadecanoate for diesters.

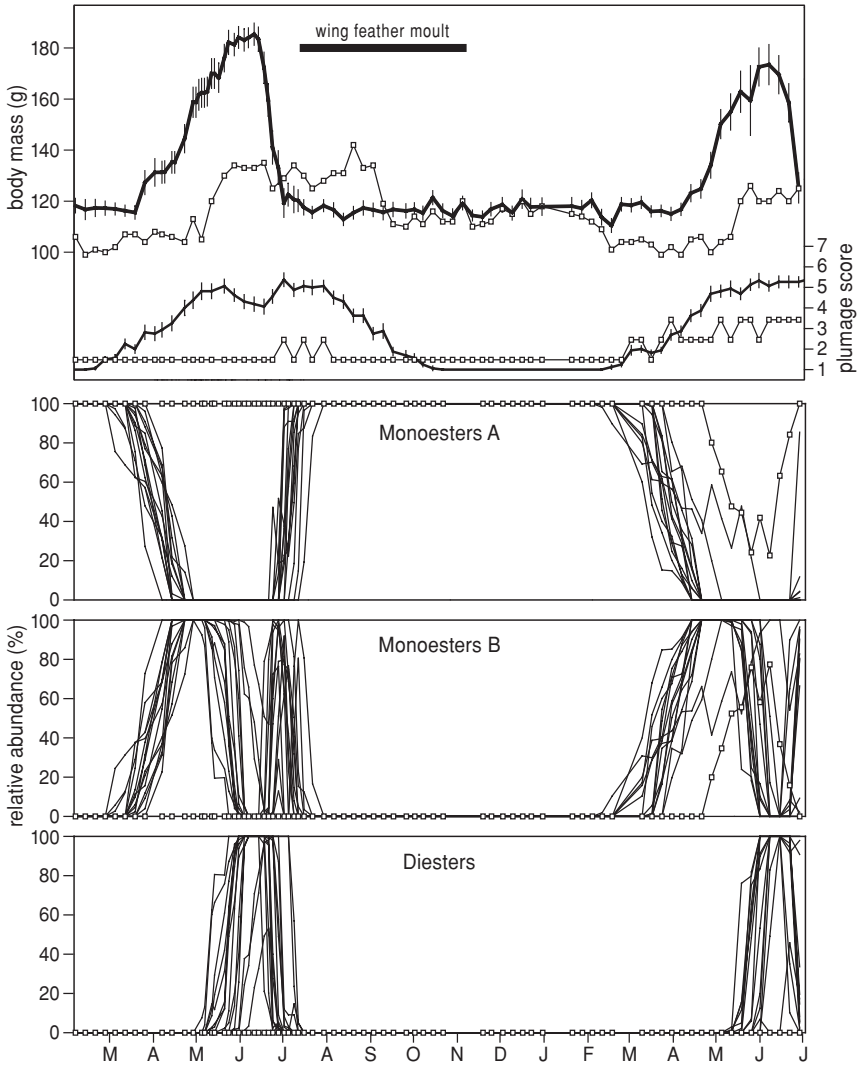


Figure 4.2 Seasonal changes in body mass, primary molt, the amount of summer plumage (indicated by plumage score) and preen wax composition in 15 individual red knots kept in outdoor aviaries in the Netherlands between March 2002 and July 2003. Averages and standard errors (vertical lines) of body mass and plumage score are depicted. For primary molt the range during which molt took place in any individual is indicated with a thick horizontal line. The percentage of monoesters A, monoesters B and diesters in preen gland secretions throughout the experiment are depicted in the lower three panels. Each line represents an individual bird. The lines with white squares indicate body mass, plumage score (upper panel) and preen wax composition (lower panels) of individual #4462 that was in poor condition.

began in August. The birds also began replacing their primary wing feathers at this stage. This is typical for red knots that forego a breeding season and spend the summer in Western Europe (Boere 1976). An individual red knot that was in a poor physical condition refrained from going into molt and did not gain body mass in anticipation of a migratory flight (#4462; fig. 4.2).

Seasonally changing preen wax composition of wild and captive red knots under natural photoperiods

The annual cycle in preen wax composition in wild red knots is depicted in Figure 4.3b. Between the end of July and mid-April, a period of approximately nine months, wild red knots secreted monoesters mixture A. This includes mainly birds in their wintering grounds but also 18 birds sampled in Brazil that were fuelling up for the first part of a migratory flight to Delaware Bay (USA) at the end of April (Piersma *et al.* 2005). Red knots in Delaware Bay in May fuelling up for the final long-distance flight to the High Arctic breeding area secreted only monoester B. From early June onwards, all red knots caught on the High Arctic breeding areas secreted mainly diester preen waxes, as has been described for 19 sandpiper species, including red knot (chapters 2 and 3). Diesters continue to be secreted during the period of incubation. After incubation, at hatch, the preen wax composition shifts back to the monoester type. Red knots sampled just after hatch in mid July secreted preen waxes consisting of mainly diesters with fractions of either monoesters A or monoesters B. None of the investigated individuals in June or July secreted a pure mixture of monoesters B only. After a short transition period in July on the breeding grounds during which preen wax mixtures of diester with monoesters A or B were secreted, birds secreted a pure mixture of monoesters A for nearly the entire nonbreeding period from mid July until mid- April (fig. 4.3b).

The three preen wax mixtures were also secreted by captive red knots. Inter-individual variation in the chemical composition of the three preen wax mixtures was not found, neither in captive or wild red knots. The annual cycles in preen wax composition of the captive red knots were synchronized between individuals (fig. 4.2) and similar to an 'average annual cycle' of wild birds (fig. 4.3). Monoesters A were secreted during a period with on average no changes in body mass (fig. 4.4). When the birds started to put on weight in spring, the shift to monoesters B took place. During the short period of highest weekly mass gain, birds secreted only monoesters B. During the mass plateau, when on average no weight was gained or lost, the shift in preen waxes to diester type took place (fig. 4.4). After this period of stable body mass, the captive birds lost weight by fasting for several weeks. It is during this period that the captive birds secreted pure diester preen wax (Figs. 4.2 and 4.4). The captive red knot (#4462) in poor

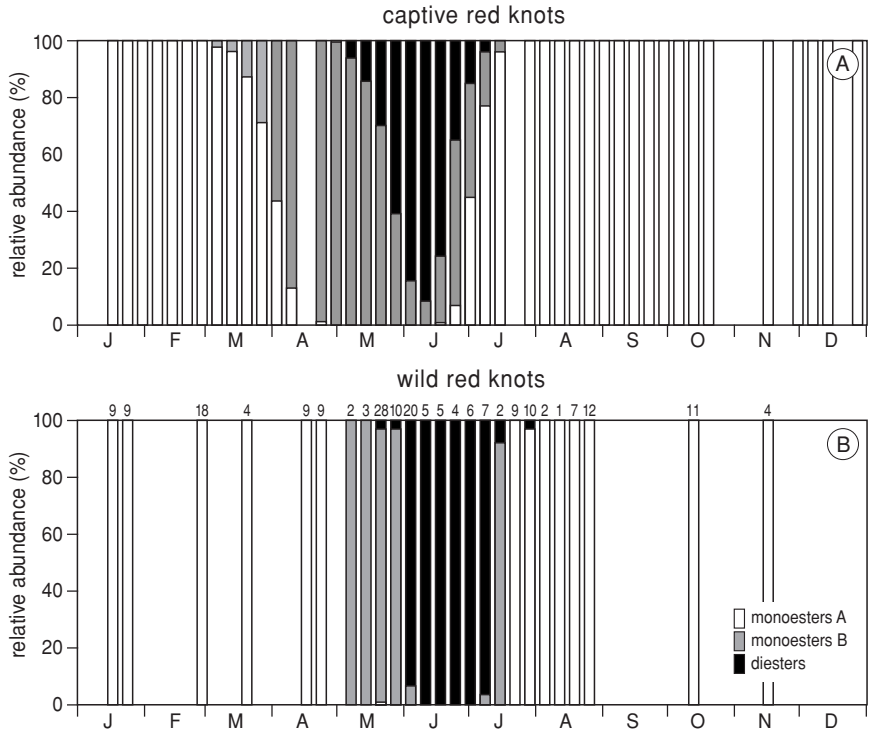


Figure 4.3 Preen wax composition throughout an annual cycle in captive (A) and wild (B) red knots experiencing a (semi) natural photoperiod. The average percentage of monoesters A, monoesters B and diesters are shown per week. On top of each stacked bar sample sizes are depicted in wild red knots. For the captive red knots, sample size is fifteen (individual #4462 was left out of this analysis).

physical condition did not secrete diester preen waxes and also less often secreted monoesters B than the other birds (fig. 4.2).

Despite the overall similarity between the annual cycle in preen wax composition of free-living red knots and the sixteen birds kept in outdoor aviaries (fig. 4.3), in the captive birds monoesters A were more gradually replaced with monoesters B in approximately nine weeks time rather than two weeks in the whole wild population, after which a slow shift of on average 30 days to diesters took place, rather than the two weeks in the wild (fig. 4.3). Diester preen wax secretion in June and July persisted longer in wild red knots (4-6 weeks) than in the captive individuals (on average less than one week) too.

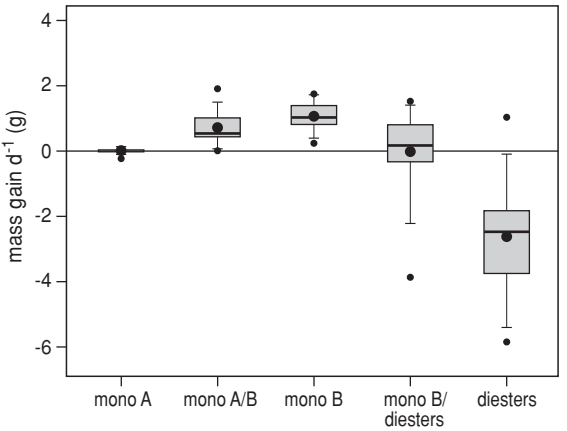


Figure 4.4 Average weekly mass gain or loss of 15 red knots in outdoor aviaries experiencing the local light regime in the Netherlands during periods when monoesters A ('mono A'), mixtures of monoesters A and B ('mono A/B'), pure monoesters B ('mono B'), mixtures of monoesters B with diesters ('mono B/diesters') and pure diesters were secreted. Body mass of the 15 birds was weekly measured when preen wax samples were collected as well. The boxes enclose 50% of the values and vertical lines indicate the range. Black dots represent the average values, the dividing lines the median. Individual #4462 was left out due to a bad condition, and the (short) period between end July – August during which mixtures of diesters with monoesters were secreted was ignored to be able to read this graph as a time series. 'mono B/diesters' thus only includes the period before complete diesters mixtures were secreted.

Phenotypic flexibility of food-restricted red knots

The birds that received only 6 hrs food per day showed lower maximal body mass during the spring fueling period (t-test, $t_{24} = 8.56$, $P < 0.001$; fig. 4.5). Five individuals, however, still gained weight and were substantially heavier during some time when they were food-restricted than before they were food-restricted (average maximal weight gain: 22.2 g, range 16 – 30 g; fig. 4.5) compared with six birds that did not (average maximal weight gain: 4 g, range –4 – 9 g). Also contour feather molt, indicated by the change in plumage scores, remained absent or was less well pronounced compared with birds that received food *ad libitum* (maximal plumage score, t-test, $t_{24} = 6.62$, $P < 0.001$); only three birds showed an increase in plumage score by at least 2 units during the period of food restriction, of which two birds had a maximum plumage score of 3, and one of 5.

Six of the eleven food-restricted birds secreted diester preen waxes in spring, and only two of them secreted pure diester preen waxes for a short period (fig. 4.5). Also, the shift back to monoesters A occurred earlier than in the control birds that received food *ad libitum* (first day after spring mass peak with more

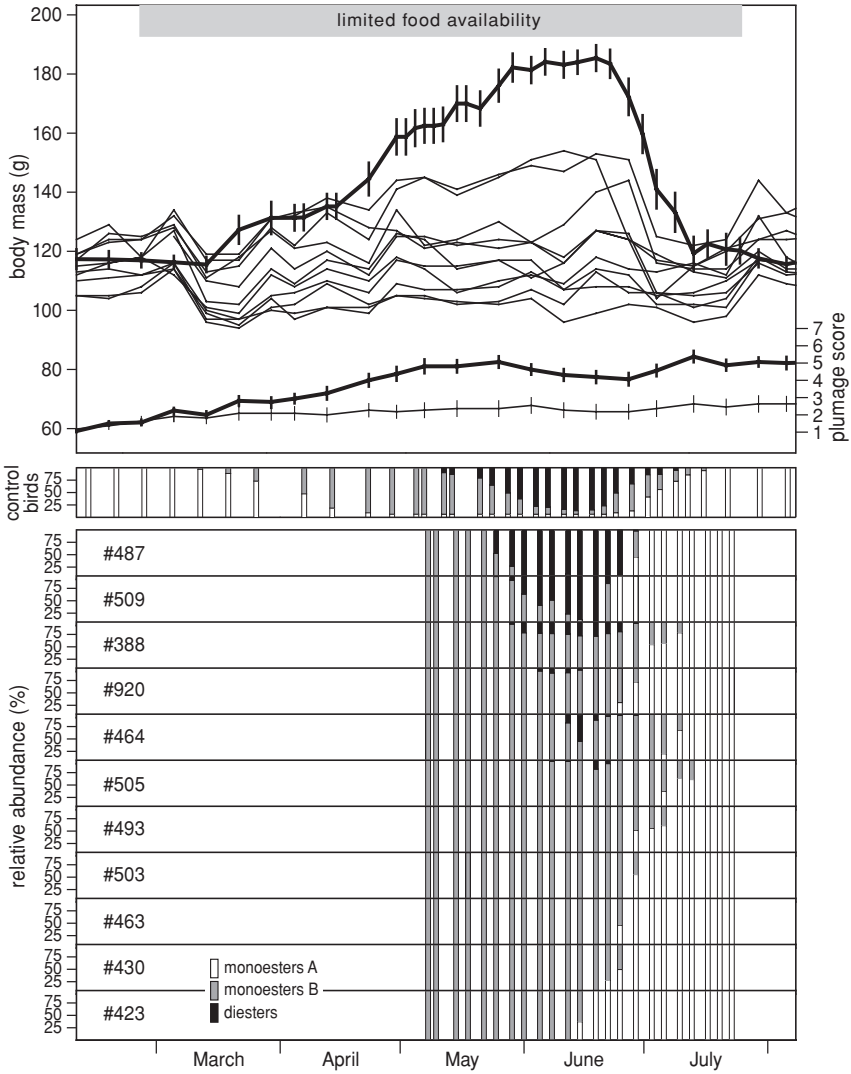


Figure 4.5 Body mass and plumage score of eleven red knots that were food restricted during spring 2002. The period during which birds were food restricted is indicated with a gray horizontal bar. The average development of body mass and plumage score of the red knots in the outdoor aviaries that received ad libitum food is depicted with thick lines, as a reference. Vertical lines indicate standard errors around the average. The percentage of monoesters A, monoesters B and diesters in preen gland secretions throughout the experiment is shown for each individual bird with stacked bars that complete 100% each. The average seasonal changes of preen wax composition of the control birds that received ad libitum food are depicted as a reference.

than 50% monoesters A, t-test, $t_{24} = 2.51$, $P = 0.019$). The timing of phenotype changes, if these occurred, did not differ from birds that received food *ad libitum*, but there was less expression in the food-restricted birds (fig. 4.5).

The degree to which diester preen waxes were secreted, indicated by the maximal percentage of diesters during the treatment, was not correlated with the maximal body mass ($F_{10,1} = 0.006$, $P = 0.94$, $R^2 = 0.0006$) or plumage score ($F_{10,1} = 0.67$, $P = 0.43$, $R^2 = 0.069$). When we ranked individuals on the basis of the amount of expression of mass peaks and pre-alternate molt, there was no correlation between the degree of expression of mass peak and molt and the maximal percentage of diesters secreted ($R_S = –0.235$, $P = 0.50$; fig. 4.6).

Phenotypic flexibility of red knots experiencing a constant photoperiod

As shown before for *canutus* red knots (Cadée *et al.* 1996), seasonal mass and molt changes of red knots (*C.c. islandica*) that had experienced a constant photoperiod for more than four years became free-running and deviated from natural annual cycles in these traits (fig. 4.7). The annual cycles in preen wax composition of these red knots also strongly deviated from those experiencing seasonal cycles in day length (fig. 4.7). The timing and duration of the body mass increases differed much from those in red knots experiencing natural photoperiods and also strongly varied between individuals (Figs. 4.2 and 4.7). Some individuals showed no obvious single mass peaks during the experiment (fig. 4.7), either no peaks at all (e.g. bird # 213) or an oscillating pattern in body mass (e.g. bird # 189).

Cycles in molt were also perturbed by the constant photoperiodic regime but not in all individuals. Only five of the fourteen individuals showed more or less 'normal' cycles of plumage scores. These birds wore an alternate plumage, indicated by plumage score scores of 5 or more, during peak body mass and a basic plumage (plumage score 2 or less) when body mass was low and stable. As in red knots experiencing seasonal photoperiodic regimes, wing molt occurred after the peak in body mass. Contour feather molt followed the mass peaks in some birds, just as captive red knots under natural photoperiodic conditions (e.g. individuals # 161 and # 295, fig. 4.7). In other individuals cycles in plumage score were perturbed. A few individuals remained in full alternate plumage throughout the period of observation (e.g. individuals # 189 and # 192).

Seasonal cycles in preen wax composition also differed from red knots experiencing natural photoperiods. The predominant preen wax mixture secreted during the experiment consisted of mixtures of monoesters A and B (fig. 4.7) in contrast to red knots experiencing natural photoperiods that secrete pure monoester A preen waxes during most of the year (fig. 4.2). None of the red knots secreted mixtures of only diesters, if diesters were secreted at all during the experiment.

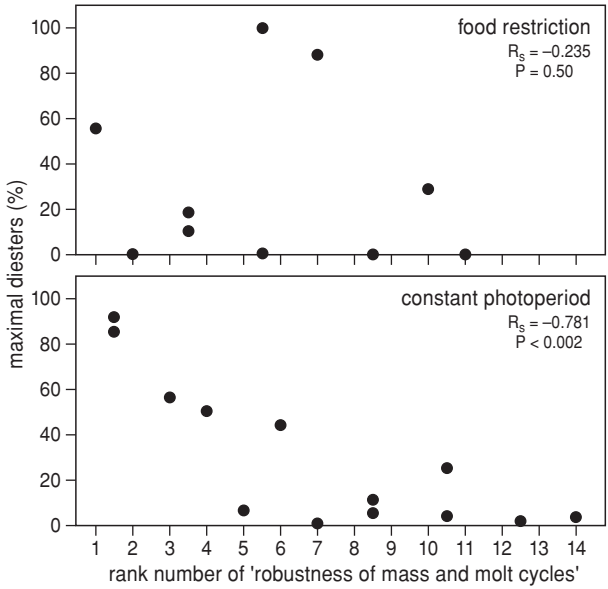


Figure 4.6 Relations between ranked robustness of individual phenotype cycles and the maximal percentage of diesters measured in birds that were given limited access to food or were put in a constant photoperiodic regime. Individuals that showed clear peaks in body mass, molted from a basic to an alternate plumage in close temporal synchrony with body mass peaks and also molted their wing feathers shortly after the peak in body mass, i.e. the birds that had the most robust organization of phenotypic cycles, received the highest rank. If on the basis of these subjective criteria, no rank could be appointed between individuals, these birds received the same (intermediate) rank.

The timing of diester preen wax secretion in those 9 of 14 birds that did secrete diesters was linked with their peak in body mass (Figs. 4.7 and 4.8) and therefore also occurred between September and January instead of May-June and was, in contrast to the birds in natural photoperiods, not synchronized between individuals (fig. 4.8). Changes in preen wax composition from monoesters to diester preen waxes persisted only in those few individuals that also showed clear cycles in body mass and molt (e.g. individual #295 and #161; fig. 4.7). However, also in these individuals no pure diester preen waxes were secreted and in some individuals a clear shift to diesters was absent even though they showed clear cycles in mass and molt (e.g. individual #178 and #220; fig. 4.7). When individuals were ranked on the basis of the expression of body mass and molt, individuals with the highest scores also secreted the highest percentage of diesters (R_S = –0.781, $P < 0.002$; fig. 4.6).

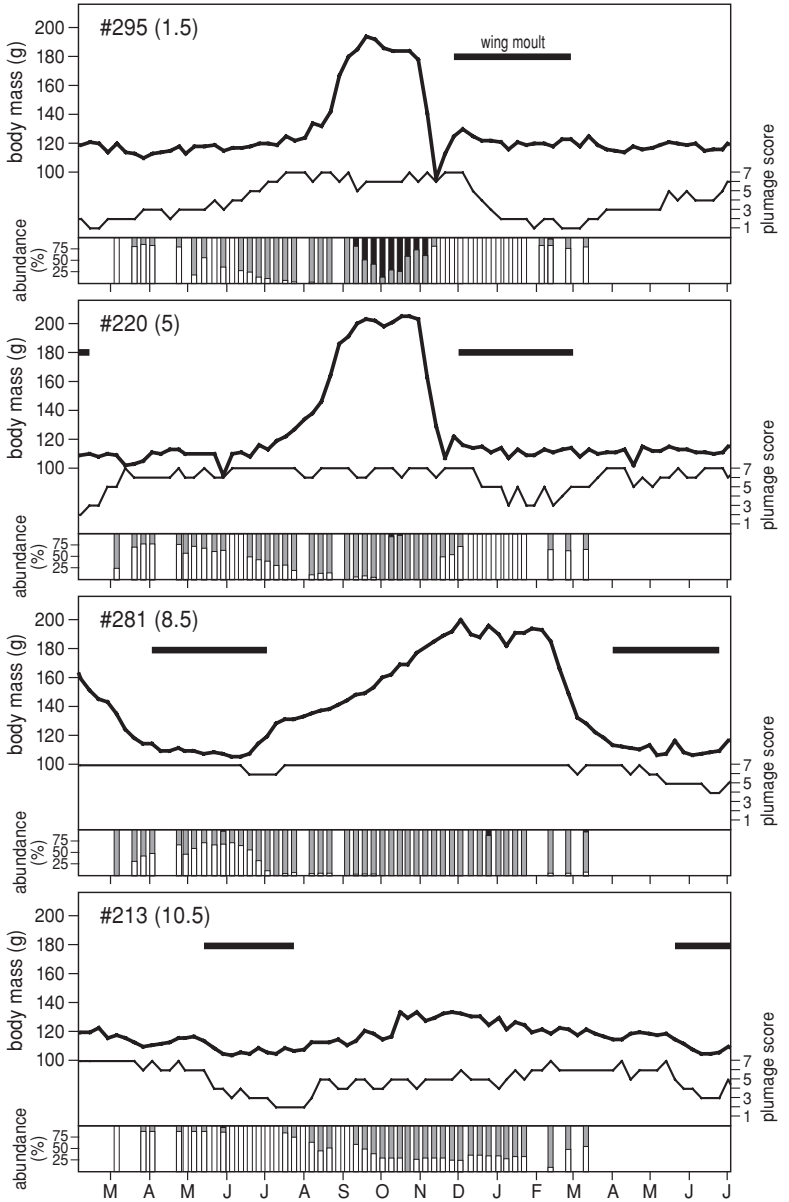
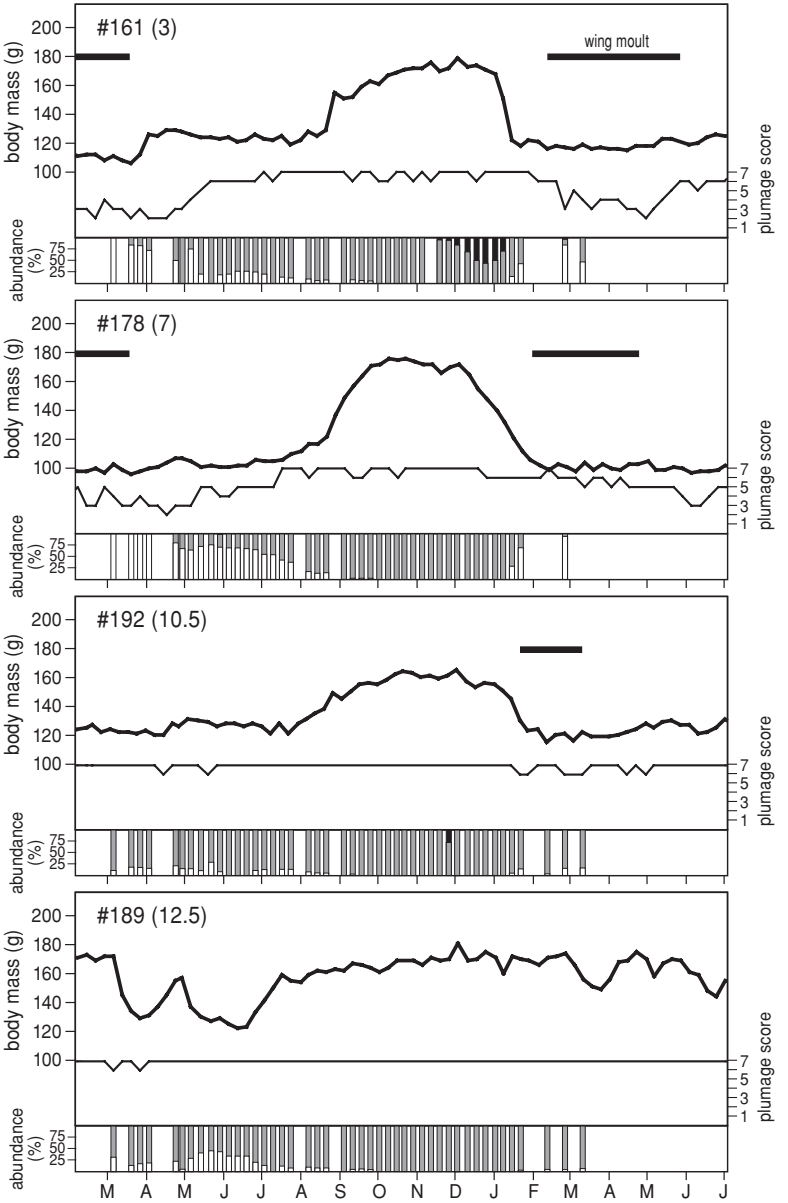


Figure 4.7 Seasonal changes in body mass, primary wing feather molt, the amount of summer plumage (score between 1 and 7) and preen wax composition in eight red knots kept in aviaries indoors with a constant photoperiod (12 hrs light, 12 hrs darkness) between 4 February 2001 and 4 July 2002. The percentage of monoesters A, monoesters B and diesters in



preen gland secretions at a given date is shown for each individual bird throughout the experiment with stacked bars that complete 100% each. The eight of fourteen individuals shown here represent different 'robustness' of seasonal phenotypic cycles and are ordered by rank (see text for the criteria used). Assigned ranks are shown between brackets behind the number of the individual bird.

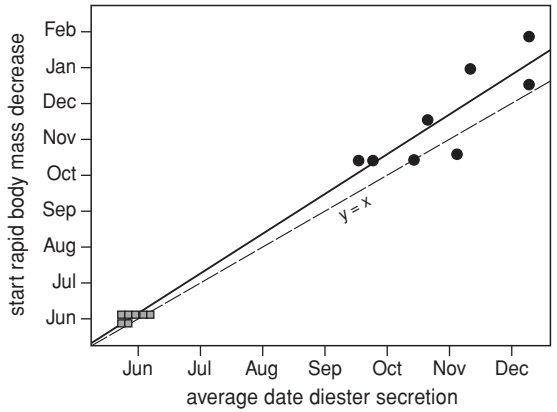


Figure 4.8 Relation between time of diester preen wax secretion and the end of the mass peak for captive red knots that experienced the natural photoperiod in The Netherlands (gray squares) and in a constant photoperiod (12 hrs:12 hrs L:D; black dots). The time of diester secretion was the date at which maximal amounts were secreted in birds in a constant photoperiod and the average between the first and last day that pure diester mixtures were secreted for birds in outdoor aviaries. The line represents the regression between date of diester secretion and the start of rapid body mass decrease for the birds in constant photoperiod only. The dashed line indicates where the average date of diester secretion occurs at the same date as the start of the body mass decrease. Five individuals kept in a constant photoperiod showed no obvious peak in body mass or did not secrete diesters and could therefore not be included in this analysis.

Discussion

Constraints on wax ester shift

Despite not being able to migrate and reproduce, captive red knots exhibited seasonal phenotype cycles that were very similar to free-living birds when exposed to seasonally changing day lengths and with *ad libitum* access to food (confirming Piersma & Ramenofsky 1998 and Piersma 2002a). The list of seasonally changing phenotypic traits thereby includes preen wax composition and we must reject our first hypothesis that captive red knots should skip the expression of a trait associated with incubation, the secretion of diester preen waxes.

Also in contrast to the hypothesis of instantaneous changes if no organizational costs are involved, changes from monoester to diester preen wax took place over a protracted period of time. Complete shifts from monoester to diester preen waxes in captive birds lasted on average 30 d in captive red knots, but could be accomplished in a shorter period (e.g. 13–14 d in some individuals,

fig. 4.2). It thus appears that, like molt and pre-migratory fueling, changes from mono- to diester secretions can not be accomplished overnight. Shifts from diester waxes back to monoesters A at hatch of eggs were faster and were accomplished in 3-8 days in some individuals, suggesting that the shift to diester secretion is more constrained than the shifts back to monoester waxes. A better understanding of the biochemical pathways of monoester and diester preen wax synthesis, may yield proximate explanations for these observations. An alternative explanation is that the chemical shifts in the secretions take time because the preen gland is a kind of 'reservoir' of waxes, that, even if the biosynthesis of preen wax would change overnight, prevent the secretions to change instantaneously because the gland is still filled with waxes of the previous type which may limit the turn-over rate of preen wax mixtures.

Endogenously regulated expression

If the transition from one phenotypic state to another takes time, the onset of such changes should start well before the time that the new phenotype actually becomes useful. In such cases endogenous regulation of the onset of change is adaptive (Gwinner 1986, 1990, 1995, Nelson *et al.* 2002). That seasonal cycles in preen wax composition, like molt and body mass (Cadée *et al.* 1996, Piersma 2002a), are endogenously controlled is suggested by the fact that the changes in preen wax composition were retained under constant photoperiodic regimes. Such endogenous regulation ensures that red knots secrete diester preen waxes immediately after arrival on the breeding grounds, one or two weeks before egg-laying and incubation (chapters 2 and 3), and sometimes even before the last migratory flight to the breeding grounds (Piersma *et al.* 1999). The secretion of diester preen waxes in captive red knots is correlated with a strong decrease in body mass just after the body mass peak in spring (Figs. 4.4 and 4.8), which may indicate the period of flight in wild birds. This would be consistent with Ramenofsky & Wingfield (2006), who argue that long-distance migratory birds have a large degree of overlap between their physiological preparations for migration and for breeding. Some redundant (energetic of functional) costs of diester secretion before red knots actually start incubation may then be unavoidable, but on the long term be outweighed by the benefits of being physiologically prepared in time for incubation, including a lower risk of losing a clutch to a mammalian predator (chapter 7).

Relative robustness of preen wax cycles

Phenotype cycles of birds in constant photoperiodic regimes showed considerable inter-individual differences and in some individuals disappeared ('faded away') completely. The timing of different phenotypical traits became desynchronized with

sometimes clear cyclicity in one, but not in other, phenotypical traits. This phenomenon has been shown before in birds deprived of seasonal cues (passerines: Gwinner 1986; red knots: Cadée *et al.* 1996). Consistent with our second hypothesis, in both treatment groups seasonal cycles in wing molt and body mass persisted in more individuals than did contour feather molt and preen wax composition. This suggests that the different traits are activated by different endogenous mechanisms, i.e. that the traits are modular (cf. West-Eberhard 1989) and that these mechanisms differ in 'robustness'. The lack of correlation between expression of different traits in food-restricted birds supports this view. In contrast, in red knots exposed to a constant photoperiodic regime, the expression of diester secretion was highest in individuals that still showed the most coherent phenotype cycles (fig. 4.6).

Even though diester secretion changes in preen wax composition are endogenously regulated and can not be changed as instantaneously as we predicted, this trait may still be more flexibly organized than are molt and seasonal mass changes. After all, transition times of preen wax composition from one to another state are still relatively short compared with the other traits. It may be adaptive for animals to not completely advance seasonal cycles of traits when this is functional during reproduction only, such as changing preen wax composition from mono- to diesters. Reproduction in long-lived species like red knots does not occur every year due to predation of clutches or because bad environmental (food) conditions do not enable a migratory flight to the breeding grounds (D.I. Rogers pers. comm.). In such non-breeding years it would be adaptive if changes of traits associated with reproduction could be turned off or not switched on at all. Phenotype changes that enhance survival, wing molt for example, should always continue and therefore be more robust.

Acknowledgements
Maarten Brugge and Bernard Spaans took care of all captive birds, weekly measured body mass and molt of the birds and helped with the preen wax sampling. Help in the laboratory came from Muriël van den Anker, Soledad van Eyk, Amy Schneider and Maaike Versteegh. Marianne Baas and Michiel Kienhuis provided technical assistance with the gas chromatography.

CHAPTER 5

Plumage reflectance is not affected by preen wax composition in red knots *Calidris canutus*

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Journal of Avian Biology 35: 405 – 409

ABSTRACT

It has recently been shown that sandpipers (Scolopacidae) abruptly switch the chemical composition of their preen gland secretions from mono- to diester waxes just before the period of courtship. The timing and context of the shift suggested that diesters could provide a visible quality signal during mate choice. We used captive red knots *Calidris canutus* to test whether mono- and diester preen waxes affect the light reflectance ("colour") of the plumage. We also determined light absorbance spectra of the two wax types. The reflectance of breast feathers of the breeding plumage was measured with spectrophotometry when birds secreted monoesters and six weeks later when they secreted diester preen waxes. Light reflectance was also measured after removing the mono- and diester waxes from the plumage with a solvent. The results show that: (1) diester preen waxes absorb more light, especially ultraviolet (UV), than monoester preen waxes, but that (2) the compositional shift in the preen waxes did not change plumage reflectance and, (3) the removal of preen waxes did not change the reflectance of the plumage within the light spectrum assumed visible to birds (320–700 nm). This is not consistent with the idea that compositional shifts in the preen waxes of red knots have a visual function.

Introduction

Plumage colouration of birds has an important signalling function in the context of mate choice and during conflicts (Andersson 1994). Sexual selection through mate choice favours brightly coloured plumages (e.g. Hill 1991). The reliability of bright plumage traits as quality signals is related to the potential costs associated with the acquisition and maintenance of a conspicuous plumage (Zahavi 1975). Conspicuous plumages may also attract predators (Butcher & Rowher 1989). The trade-off between impressing potential mates and attracting predators is generally believed to be the underlying mechanism for many bird species to carry conspicuous plumage during the breeding season only.

Complete replacement of contour feathers requires a moult of several weeks and is therefore not a quick and flexible mechanism to adjust plumage colouration to accommodate abruptly changing needs. To avoid costly and time consuming moult, male rock ptarmigans *Lagopus mutus* soil their conspicuous white breeding plumage with dirt as soon as mating has taken place to increase crypsis (Montgomerie *et al.* 2001). The birds clean off the dirt when mating opportunities become available again, e.g. after a clutch is lost. Similar behaviour has been observed in bearded vultures *Gypaetus barbatus* that actively stain their plumages with orange soil containing iron oxide (Negro *et al.* 1999).

Greater hornbills *Buceros bucornis* use their preen gland secretions to yellow plumage areas that are used for signalling during threat displays (Elder 1954, Del Hoyo *et al.* 2001). Piersma *et al.* (1999) suggested that the diester preen waxes produced just before and at the beginning of the breeding season by red knots *Calidris canutus* may also have a cosmetic function. For most of the year the uropygial gland secretions of red knots consist of monoesters. A more detailed study of 19 sandpiper species (chapter 2) showed that shifts from mono- to diester preen waxes are common to all of these species and that diester preen waxes are continuously secreted throughout the period of incubation by the incubating sex only. In red knots both of the sexes incubate and both sexes secrete diester preen waxes during incubation (chapters 2 and 3). Therefore, the secretion of diesters is better correlated with incubation than with courtship and display.

For the diester preen waxes to function as avian cosmetics, shifts in preen wax composition should bring about a visual change of the plumage as seen by the birds themselves. We could not see a difference in plumage colouration of red knots with a shift in preen wax composition. However, most diurnal birds can detect wavelengths in the UV portion of the spectrum down to 320 nm, which are invisible to humans (Burkhardt 1989, Bowmaker *et al.* 1997). Therefore, a possible change in plumage appearance in red knots could remain unnoticed by human investigators (Bennett *et al.* 1994). In addition, red knots and other sand-

pipers breed in (sub)arctic regions with long days of sunshine and snow that increases the UV radiation load (Caldwell *et al.* 1980). Absorption of UV light by diester preen wax could protect feathers against potential damage and also affect reflectance of the plumage. We used spectrophotometry to determine both light absorption of mono- and diester preen waxes and the effect of both preen waxes on the reflectance of the contour feathers of red knots in breeding plumage.

Materials and methods

The birds and their preen waxes

We used six captive knots kept in two separate outdoor aviaries. Captive red knots exposed to the Dutch photoperiodic regime undergo semi-natural annual cycles in body mass and moult (Piersma & Ramenofsky 1998). Contour feather moult from a grey winter plumage to a rusty red breeding plumage takes place between March and May. The captive red knots also show annual cycles in preen wax composition (chapter 4). Preen wax was sampled twice a week by massaging the feathered nipple of the preen gland with a cotton bud. The wax was dissolved in ethyl acetate to a concentration of 1 mg wax/ml. The composition of the intact waxes was determined with capillary gas chromatography as described elsewhere by Dekker *et al.* (2000). The preen wax composition of all individuals was analysed every week so that we were able to compare reflectance of the breeding plumages of red knots before and after the chemical shift from mono- to diester preen wax.

Effect of presence of different preen waxes

The absorption spectra (190–900 nm) of mono- and diester preen waxes were obtained using a Uvikon 940 spectrophotometer. As a reference we used pure ethyl acetate. Absorbance spectra of six monoester and four diester samples were measured. The analysis of the absorption spectra was restricted to wavelengths above 290 nm only, as solar ultraviolet radiation shorter than 290 nm is absorbed in the high atmosphere by ozone and oxygen and absent in the earth's atmosphere (Gates 1966).

Reflection spectra of the red knots' plumages were measured on 9 May 2002 when the birds were secreting monoester preen waxes, and on 24 June 2002 when they were secreting diester preen waxes. Preen waxes on feathers were always the same as the waxes harvested from the uropygial gland (M. Dekker unpubl. data). Therefore, we are confident that the wax on the plumage had the same composition as the wax sampled from the uropygial gland, especially as the shift to diester preen waxes took place 1–3 weeks before the second reflectance

measurements. As prealternate moult of contour feathers was completed before measurements were performed, possible differences in reflectance of birds between dates could not be attributed to replacement of feathers. Both on 9 May and on 24 June 2002 reflection spectra of the rusty red breeding plumage of the birds were measured before and after removal of preen waxes from the breast feathers. We removed waxes with a solvent (ethyl acetate) on cotton wool after the initial reflectance measurements. The cotton wool often turned yellowish during this treatment. This colour was similar to a cotton bud after massaging the preen gland nipple. Several minutes after this treatment we checked whether the solvent had completely evaporated by the absence of the smell of ethyl acetate before measuring the reflectance the second time.

To check whether with this treatment all of the preen waxes were effectively removed from the breast feathers, three red knots that were not used in the experiment underwent this treatment twice in a row. All waxes were extracted with ethyl acetate from the cotton wool and analysed by gas chromatography conform the analysis of the preen wax samples. We expected to find waxes after the first treatment but not after the second treatment. The gas chromatograms showed that after both treatments many more unidentified hydrophobic compounds were extracted from the plumage than preen waxes. Only a small fraction (less than a percent) of all removed compounds consisted of preen waxes.

Reflectance of the breast feathers was measured with an AVS-USB-2000 (Avantes, Eerbeek, The Netherlands) spectrophotometer, with illumination by an Avantes DH-2000 Halogen-Deuterium light source, both connected to the measuring probe by a bifurcated fibre optics cable. The measuring probe was fitted with a round plastic tube (6.35 mm inner diameter) to exclude ambient light and to keep the distance between the probe and the feathers constant. During the measurements the probe was held at a right angle to the plumage, i.e. both illumination and recording were at 90° to the feathers. Of each bird the reflectance of ten haphazardly selected spots on the breast were measured. Reflectance was expressed relative to a WS-2 white reflection standard (Avantes).

Statistical analyses

We used principal component analysis (PCA) to reduce the information contained in the absorbance and reflectance curves, which consist of many correlated reflectance values, into a few variables adequately describing the existing variation among curves (Endler 1990). We performed a PCA without factor rotation on correlation matrices of 22 absorbance values separated by 20 nm intervals between 290 nm and 710 nm and of 20 reflectance values of each curve between 320 and 700 nm. We only extracted principal components that had eigenvalues greater than 1. Non-parametric statistics (Mann-Whitney U test) were used to

analyse absorbance measurements because the principal component was not normally distributed.

Spectral analyses of the reflectance curves of the red knots' plumages were restricted to 320-700 nm, the spectral range that is likely to be visible to most birds (Maier 1994). Raw spectra were smoothed by calculating a running mean over a 20 nm interval. Subsequently, the ten smoothed reflectance curves obtained for each bird were averaged.

Results

The first principal component (PC1) of the PCA explained 96% of the variation in the absorbance curves. The loadings of PC1 were all positive and higher than 0.90 except for 290 nm which had a loading of 0.61. Absorption spectra of preen wax samples revealed that diester waxes absorbed light better than monoester waxes (Mann-Whitney U, $Z = -2.56$, $P < 0.0001$), especially in the UV (fig. 5.1).

The difference in light absorption did not result in a change of plumage reflectance with the shift from mono- to diesters in June (fig. 5.2). Reflection spectra of the breeding plumages of red knots are typical of red or chestnut-coloured feathers (Burkhardt 1989). There is no reflectance peak in the UV band (between 320–400 nm), and reflectance gradually rises with wavelength (fig. 5.2). The highest average percent reflectance occurs in the long wavelength portion of the light spectrum.

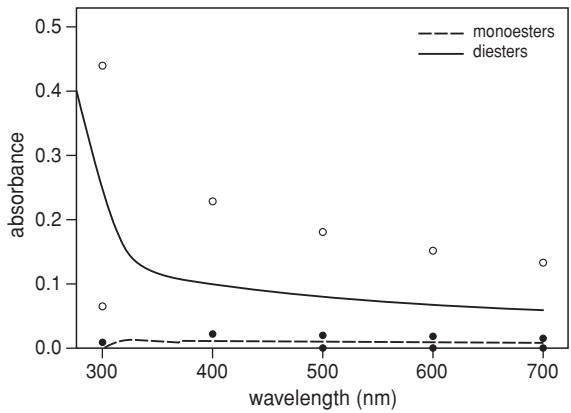


Figure 5.1 Absorption spectra of mono- (dashed line) and diester (solid line) preen waxes. Six samples of monoesters were analysed and four samples of diesters. Dots indicate 95% confidence intervals at 100 nm increments for monoesters (black dots) and diesters (white dots).

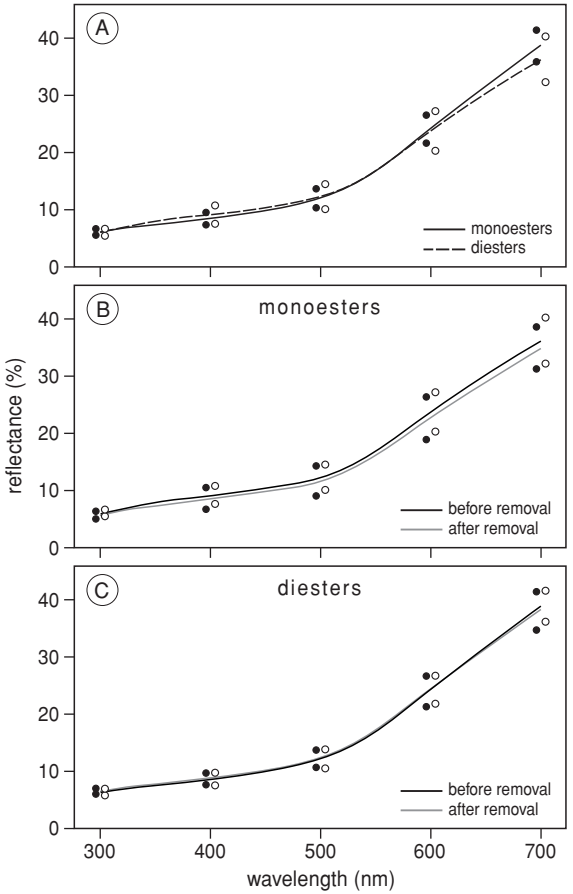


Figure 5.2 Pairwise comparison of reflectance of breasts of six red knots in breeding plumage with monoester and diester preen waxes (A), before and after removal of monoester preen waxes (B), and before and after removal of diester preen waxes (C). Dots indicate 95% confidence intervals at 100 nm increments (staggered for clarity reasons) of reflectance curves of birds with diester (black dots) and monoester (white dots) preen waxes in (A) and of birds of which waxes have been removed from the plumage (black dots) and of birds before preen wax removal (white dots) in (B) and (C).

The PCA adequately described the variation among measured reflectance curves. PC1 explained 92.0% and the PC2 explained 7.0% of the total variation in reflectance values among reflectance curves. PC1 had strong positive loadings (between 0.86 and 1.00) of all wavelengths along the whole 320–700 nm range, which indicated that PC1 was correlated with achromatic brightness of the

plumage. This was confirmed by a strong correlation between PC1 and average percent reflectance between 320–700 nm ($r = 0.994$, $P < 0.0005$, $n = 24$). When using PCA to summarise reflectance curves the extracted PC2 is typically associated with variation in the shape of the curves, which may be visually perceived as variation in hue or chroma (Endler 1990). PC2 showed low to moderate negative loadings (between –0.01 and –0.31) from wavelengths between 320–520 nm, and low to moderate positive loadings (between 0.04 and 0.49) from wavelengths between 540–700 nm. Thus, the plumages of birds with high PC2 scores have relatively lower reflectance between 320-520 nm and relatively higher reflectance between 540–700 nm.

A change of preen wax composition from mono- to diesters did not result in a statistically significant change in PC1 (fig. 5.2A; paired t-test $df = 5$, $t = –0.19$, $P = 0.86$). It did change the shape of the reflectance curves as indicated by the statistically significant change in PC2 (paired t-test $df = 5$, $t = 6.12$, $P = 0.002$), although the magnitude of the effect was small (fig. 5.2). The significant difference in PC2 between May and June persisted for feathers of which preen waxes were removed (paired t-test $df = 5$, $t = 4.77$, $P = 0.005$), indicating that the small change in shape of the reflectance curves was not caused by a change in wax composition from May to June.

Removal of monoester (paired t-test, $df = 5$, PC1: $t = 0.72$, $P = 0.50$; PC2: $t = –0.44$, $P = 0.68$; fig. 5.2B), or diester preen waxes (PC1: $t = 0.07$, $P = 0.95$; PC2: $t = 1.68$, $P = 0.15$; fig. 5.2C) did not affect reflectance. This again suggests that the differences in the shape of the reflectance curves, as indicated by the significant change in PC2 between May or June, are probably not caused by the change in preen wax composition between those dates.

Discussion

Although diesters absorb more light than monoesters, this did not affect reflectance of plumage with a coating of mono- or diester preen wax. Light reflectance of the red knots' plumages is relatively little for wavelengths below 500 nm, even after preen waxes were removed, suggesting that feathers themselves absorb much light in this part of the light spectrum. This would imply that with a change from a coating of mono- to diester waxes on the feathers, less light of short wavelengths such as UV is absorbed by the feathers and more by the waxes without a net effect on plumage reflectance.

Plumage brightness of the red knots did not differ between May (monoesters) and June (diesters), but a small change in the shape of the reflectance curves between May and June was observed (fig. 5.2). We do not know if the magnitude of

the effect is large enough to be distinguishable to red knots. Because feathers of which preen waxes were removed showed the same difference between May and June as feathers with preen waxes of different composition, the shift in the shape of the reflectance curves is unlikely to be caused by the shift in preen wax composition. The reflection spectra of the feathers themselves did change slightly, probably as a result of feather wear (Örnborg *et al.* 2002).

Because we removed a large amount of hydrophobic compounds (several mg) from the breast feathers with the ethyl acetate, we believe that the lack of a change in visual appearance of red breast feathers of red knots indicates that the colour of feathers is mainly caused by the feather pigments. The layer of waxes and other compounds on feathers is probably too thin to cause a detectable change in light reflectance. This may also explain the discrepancy between the yellowish colour of smears from the preen gland and the lack of change in visual appearance of the plumage after preen wax removal. This is not consistent with the idea that colour changes during preen wax shifts play a role in the visual communication in red knots (Piersma *et al.* 1999), but there is still the possibility that the different preen wax compositions induce a different shine on the plumage (cf. the effect of shoe polish on leather).

The increased absorbance of light, and especially UV, by diesters could be functional. Diesters may better protect feathers against harmful UV radiation than monoesters, but this idea needs further testing. Although red knots are subjected to 24 hrs of sunlight on their breeding grounds and the presence of snow may increase the UV radiation load, arctic regions generally have a lower UV radiation load than temperate or tropical wintering grounds of red knots (Caldwell *et al.* 1980). It is also unclear why diester preen waxes are only secreted by incubating individuals (chapter 2). Currently, two other hypotheses are being investigated: (1) less volatile diester preen waxes diminish the scent of incubating birds and hence increase 'olfactory crypsis' (chapter 2), and (2) diester preen waxes protect birds' feathers more efficiently than monoesters against feather degrading bacteria, fungi and lice during periods during which they spend much time on the nest.

Acknowledgements
We thank Theunis Piersma, Danny Rogers, Matthew Berg and Jaap Sinninghe Damsté for critical comments on earlier drafts. Marten Staal helped with the light absorbance measurements. The work is financially supported by ALW grant 810.34.003 to Theunis Piersma and Jaap Sinninghe Damsté of the Netherlands Organisation for Scientific Research (NWO) and ALW grant 810.67.022 to Jan Komdeur.

Box C Sudden colour changes in female lapwings at the start of incubation are unrelated to preen wax changes

Jeroen Reneerkens, Joop Jukema & Theunis Piersma

Lapwings *Vanellus vanellus* have always played an important folkloristic and financial role in the daily lives of many Dutch farmers. They are typical and numerous meadow birds whose eggs were collected for consumption and sale, especially in the province of Friesland, where some tradesmen sold in the 1960's eight to ten thousand lapwing eggs per day in early April (van Dijk 1967). Clearly, there was money to earn with the trade in lapwing eggs and the early egg collectors had developed a fine nose to find their clutches and became very skilled in recognising the essential signs to recognise whether birds had laid eggs. One of such characteristics is the notion that the plumage of female lapwings becomes dull as soon as they start to incubate. As lapwings do not moult just before the start of incubation, and certainly would not be able to replace their feathers within the short period over which the change in colour was observed, there had to be another way that enabled the birds to change plumage characteristics.

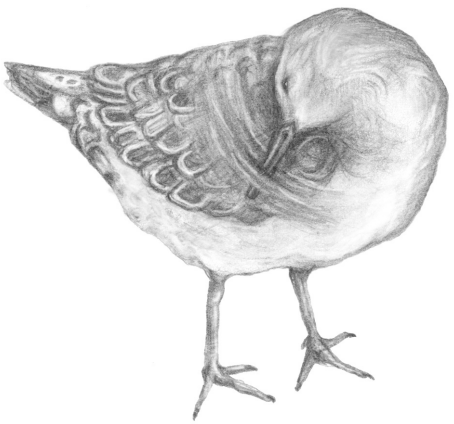
A logical first suggestion was that a sudden change of preen wax composition would cause this apparent change in plumage colouration or brightness. First we needed to find out whether the composition of the preen wax composition in lapwings changed shortly before the start of incubation, as in other shorebird species (chapter 2), and whether this only happened in females. With the help of Frisian bird catchers we collected preen wax samples of 43 individual lapwings (both males and females) in different months thoughout the year. We focused on the period just before and during incubation but also collected samples outside the breeding period (eleven samples from September - December). The samples were analysed using gas chromatography and simply interpreted by judging the peak pattern that is typical for monoester mixtures or diester mixture (see chapter 2 for methodology).

In contradiction to our expectations, the gas chromatograms of the preen wax samples of lapwings all looked like typical gas chromatograms of mixtures of monoesters such as they are secreted by other shorebirds. So, time of the year, incubation, sex nor age had any visible effect on preen wax composition. We thus may conclude that the observed change in plumage brightness by experienced collectors of lapwing eggs can not be explained

with a change in preen wax composition (Jukema *et al.* 2003b). Perhaps, lapwings bath and preen less once they start incubation, which may explain the observation of change in plumage appearance (cf. Montgomerie *et al.* 2001).

That lapwings are not the only shorebird in which preen wax composition seems invariable throughout the year became clear when we also studied Kentish plovers *Charadrius alexandrinus* and dotterels *Charadrius morinellus*. The preen wax composition of thirteen Kentish plovers caught in Oregon, USA, between 30 May – 29 July 2001 all appeared to consist of monoesters only, even though eleven of the birds were caught during incubation and two after hatch. Similarly, a comparison of gas chromatograms of preen wax secreted by twelve adult dotterels caught during spring migration and incubation near Medusa Bay, Siberia, with a single juvenile individual vagrant caught in The Netherlands on 15 November 2000, showed no apparent difference. In all cases the gas chromatograms looked similar to those of pure monoester A mixtures of red knots (see chapter 4), but GC-MS of the hydrolysed wax should proof that the preen wax indeed consisted of monoesters only. In any case, the simple analyses performed so far clearly indicate that the wax composition is invariable in these three species, in contrast to most other shorebird species investigated so far (chapter 2, Reneerkens *et al.* 2006b).

Interestingly, a phylogenetic analysis of many shorebird species suggests that lapwings, Kentish plovers and dotterels are closely related (A.J. Baker pers. comm.). Although Kentish plovers are usually placed in phylogenetic clades with other small plovers such as ringed plover *Ch. hiaticula* and little ringed plover *Ch. dubius*, in which preen wax composition shifts from mono- to diesters during incubation (Reneerkens et al 2006b), the lack of seasonal variation in preen wax composition does indeed suggest that this species is more related to the lapwings. This finding of invariable preen wax composition in a few closely related species that live in different habitats and have different life histories, suggests that seasonal changes in preen wax composition have evolved at the node of speciation and that these are evolutionary conservative (see also box B).



CHAPTER 6

Seasonally changing preen wax composition: red knots' flexible defense against feather-degrading bacteria?

Jeroen Reneerkens, Maaike A. Versteegh, Amy M. Schneider, Theunis Piersma & Edward H. Burtt Jr.

Submitted

ABSTRACT

During incubation, ground-breeding sandpipers such as red knots *Calidris canutus* create a warm, humid microclimate in the nest, conditions that favor the growth of feather-degrading bacteria present in their plumage. Just before incubation, the composition of waxes secreted by the uropygial gland of red knots and other sandpipers changes quickly and completely from a mixture of only monoesters to a mixture of only diesters. We hypothesized that the change in composition of the preen wax helps protect the plumage against feather-degrading bacteria. We tested the hypothesis by studying growth of the feather-degrading bacterium *Bacillus licheniformis* (which has been positively identified in the plumage of breeding and non-breeding red knots) on the feathers of red knots with natural amounts of mono- or diester preen waxes. The removal of preen waxes from feathers resulted in faster degradation of the feathers, confirming earlier studies that preen wax inhibits growth of feather-degrading bacteria. However, the rate of degradation of feathers with preen wax based on diesters did not differ from that of feathers with preen wax based on monoesters. We argue that preen waxes protect feathers by forming a physical barrier to microbes rather than by chemical properties of the waxes, such as acidity.

Introduction

Most birds apply waxes secreted by their preen gland onto their feathers as part of maintenance behavior (Jacob & Ziswiler 1982). The secreted preen waxes are species-specific, complex mixtures usually consisting of wax esters (i.e., fatty acids condensed with alcohols; Jacob 1976, Sweeney *et al.* 2004). The species specificity of these waxes suggests that different habitats subject birds to different selective forces (e.g., humidity, predation pressure, UV radiation) and may have led to the evolution of varied preen wax compositions to accommodate specific needs (Sweeney *et al.* 2004). Preen wax esters consisting of alcohols esterified to unbranched fatty acids, for example, are more water repellent than preen wax esters consisting of alcohols esterified to branched fatty acids (Sweeney *et al.* 2004), and thus might occur more often in waterbirds. Furthermore, preen wax composition sometimes varies intra-specifically based on season (Jacob *et al.* 1979, Kolattukudy *et al.* 1987, Piersma *et al.* 1999, chapter 2). Such seasonal variation in preen wax composition may be the result of different selection pressures that birds encounter in the course of an annual cycle. For example, it is advantageous to secrete less volatile wax mixtures in periods when birds are exposed to a high risk of predation by mammals that search by olfaction (e.g., when incubating a clutch in an easily accessible nest; chapter 7).

Another selection pressure that may explain variation in preen wax composition is the occurrence and density of feather parasites (Sweeney *et al.* 2004). The plumages of birds harbor a variety of bacteria, many of which are able to degrade feathers (Burtt & Ichida 1999, 2004, but see Cristol *et al.* 2005). Degradation of feathers could increase thermoregulatory costs as a result of reduced insulation, increased heat loss and, as a consequence, reduced body mass and survival (Booth *et al.* 1993, Clayton 1999). In addition, degradation of the flight feathers could reduce aerodynamic efficiency of the bird (Barbosa *et al.* 2002). Many feather-degraders are soil bacteria (Wood 1995, Lucas *et al.* 2003). Consequently, birds foraging on the ground have a higher incidence of feather-degrading bacteria than birds that forage in foliage, on bark, or in the air (Burtt & Ichida 1999). In warm, moist environments vegetative cells of feather-degrading bacteria become metabolically active and degrade feathers rapidly (Burtt & Ichida 2004). Because relatively warm, moist conditions are created in the nest scrapes of incubating shorebirds (Ar & Sidis 2002), these birds are more likely to encounter metabolically active feather-degrading bacteria than non-incubating conspecifics or bird species that nest above the ground.

We test whether a seasonal change in preen wax composition might offer specific protection against feather-degrading bacteria in a ground-nesting sandpiper, the red knot *Calidris canutus*. Preen wax has been shown to inhibit the

growth of feather-degrading and skin bacteria (Bandyopadyay & Bhattacharyya 1996, Shawkey *et al.* 2003). Just before the breeding period, sandpipers (Scolopacidae) show an abrupt shift in preen wax composition from preen waxes based on short-chained monoesters to more viscous secretions based on longer-chained diesters (Sinninghe Damsté *et al.* 2000, chapter 2). Secretion of diester preen waxes by sandpipers occurs only during the weeks when the eggs are laid and incubated and only in individuals (or sexes) that incubate (chapter 2 and 3). These two facts suggest that the chemical shift is related to some demand of incubation. We quantified the effect of preen wax composition on the growth of *Bacillus licheniformis*, a common feather-degrading bacterium found in many species of wild birds (Burtt & Ichida 1999).

Methods

Occurrence of feather-degrading bacteria in red knots

In the summer of 2003 bacterial samples were collected from the plumage of seven red knots on the breeding grounds near Zackenberg Research Station on Wollaston Forland (74º 28' N, 20º 34' W), Northeast Greenland and of 28 red knots staging in the Dutch part of the Wadden Sea, on a high tide roost on the sandbank Richel (53º 17' N, 05º 07' E). Samples were taken by wetting a sterile Dacron swab with sterile saline and rubbing it over the plumage of the birds. The swabs were re-sealed in their sterile packaging to prevent contamination and refrigerated at 5°C until processed.

Because the types of bacteria in the plumage were unknown and our goal was to identify *B. licheniformis* and other potential feather-degrading bacteria, media were chosen to accommodate different growth preferences and select for bacteria known to degrade feathers (*B. licheniformis*, in particular). These selective media included Tryptic Soy Agar (TSA), Glycerine Asparine Agar (GAA), Tomato Paste Oatmeal Agar (TPO), Yeast Maltose Agar (YMA) and Nutrient Broth Alkaline Salt solution (NBas). TSA and NBas were used as a selective medium for *Bacillus* sp.; GAA, TPO and YMA were used to isolate Actinomycetes. In the lab, the bacterial swabs were streaked across plates of TSA, GAA and TPO Media and then placed in test tubes of NBas. Plates were incubated at 37°C (TSA), or 28°C (GAA, YMA and TPO plates) for 48 hrs. After 48 h agar plates were removed from incubation and colonies were counted. Plates that did not show evidence of bacterial growth were discarded. Sterile loops were used to streak single colonies onto fresh plates of TSA and YMA media. These plates were incubated at 37°C (TSA) or 28°C (YMA) for 48 hrs. A sterile loop was used to inoculate tubes of the same media with an isolated colony and these tubes were in-

cubated for 48 hrs, as described above. The resulting tubes contained isolates of bacteria and were stored at 4°C. Media-specific keys were used to classify bacteria based on colony morphology. For example, *B. licheniformis* colonies were identified by their wrinkled, mounded appearance. Additionally, we used Gram-staining and oil immersion light microscopy to classify the bacteria from each isolate based on a positive or negative Gram stain and basic morphological characteristics, such as the rod shape of bacilli (Singleton 1997).

NBas tubes were incubated at 50°C for seven days with constant oscillation. The modified nutrient broth and high temperature favor the growth of *B. licheniformis* and inhibit the growth of most other microorganisms (Burtt & Ichida 1999). After seven days, tubes were removed and bacterial growth was assessed. If the broth remained clear, the colony was not *B. licheniformis* and the broth culture was discarded. If the broth became cloudy, bacilli were cultured by cross-streaking a loopful of the media on a sterile TSA plate and incubating it at 37°C for 48 hrs. If colonies grew, we removed one with a sterile loop and inoculated a tube of TSA, which was incubated at 37°C for 48 hrs. The resulting culture, which was stored at 4°C, was a pure isolate of *B. licheniformis* from a known red knot.

A known strain of *Bacillus licheniformis* (OWU 1455) was cultured following the procedures described above and used for comparison when identifying bacterial isolates. We did not grow control cultures of bacteria other than *B. licheniformis*. Details on preparation and identification of (feather-degrading) bacteria in feathers of red knots are given in table 6.1.

Collection of feathers and preen waxes

Feathers were collected from 16 adult (i.e. more than 2 years old) red knots that were held in outdoor aviaries exposed to the local light regime at Texel, The Netherlands. The birds were caught with mistnets at high tide roosts in the western part of the Wadden Sea and had been in captivity for 4 to 9 years at the time of feather sampling. The red knots showed annual cycles in mass, molt and preen wax composition (chapter 4) similar to free-living conspecifics. On 4 May 2005 preen wax and feathers were collected from 17 birds in full breeding plumage. On 17 June 2005 these birds were sampled again. The birds had not molted their breast feathers between sampling dates. On both dates at least 0.16 g of feathers were collected with a pair of forceps to avoid rubbing wax off the feathers. A few mg of preen gland secretions were collected by gently rubbing a cotton bud over the papilla of the uropygial gland.

Table 6.1 (right) Description of the bacteria identified in plumages of red knots. In some occasions more than one colony of bacteria was isolated from swabs of individual birds.

Ring no.	Date; location	Preen wax	Preparation[a]	Gram stain; description	Description plate growth	Bacteria
8882036	July 4; NE Greenland	Diesters	TSA-TSA	+ ; Cocci in random packets	Glistening, white, raised; cocci	*Staphylococcus* sp.
			TSA-YMA	+ ; Cocci in random packets	Small, white, raised, glistening colonies	*Staphylococcus* sp.
8882059	July 29; NE Greenland	Monoesters	NBas-TSA	+ ; Scattered rods, some in clusters	Raised, wrinkled, cream-colored, matte; spore-forming rods	*B. licheniformis*
Z023407	Aug. 29; Wadden Sea	Monoesters	NBas-TSA	+ ; Rods in net-like pattern	Raised, wrinkled, cream-colored, matte spore-forming rods	*B. licheniformis*
Z023408	Aug. 29; Wadden Sea	Monoesters	NBas-TSA	+ ; Rods arranged in net-like pattern	Raised, wrinkled, cream-colored, matte spore-forming rods	*B. licheniformis*
Z023411	Aug. 29; Wadden Sea	Monoesters	TSA-TSA	+ ; Even-numbered packets of cocci	Whitish opaque circular colonies; cocci	Unknown cocci
			NBas-TSA	+ ; Rods arranged in net-like pattern	Raised, wrinkled, cream-colored, matte; spore-forming rods	*B. licheniformis*
			TSA-YMA	+ ; Even-numbered packets of cocci	Small, white, raised, glistening colonies	Unknown cocci
Z023412	Aug. 29; Wadden Sea	Monoesters	TPO-TSA-TSA	+ ; Scattered rods	Raised, wrinkled, cream-colored, matte; spore-forming rods	*B. licheniformis*
			TPO-TSA-TSA	+ ; Scattered rods	Raised, wrinkled, cream-colored, matte; spore-forming rods	*B. licheniformis*
Z023414	Aug. 30; Wadden Sea	Monoesters	TSA-TSA	+ ; Bacilli with spores	Widespread translucent feathery colonies; two large clumps of pinkish-brown filamentous cells	Unknown
			TPO-YMA-YMA	+ ; Rods with some spore-looking structures	Actinomycetes	*Streptomyces* sp.
			TSA-YMA	+ ; Short rods scattered throughout	Actinomycetes	*Streptomyces* sp.
Z023424	Sept. 2; Wadden Sea	Monoesters	TSA-TSA	+ ; Cocci	Yellow translucent, circular colonies; cocci	Unknown cocci
			TSA-YMA	+ ; Cocci	Large, raised, yellow, glistening colonies with irregular shape	Unknown cocci
Z023426	Sept. 2; Wadden Sea	Monoesters	NBas-TSA	+ ; Rods arranged in net-like pattern	Raised, wrinkled, cream-colored, matte; spore-forming rods	*B. licheniformis*
Z023433	Sept. 4; Wadden Sea	Monoesters	TSA-TSA	+ ; Cocci, many, in even-numbered clusters	Glistening, white circular; cocci	Unknown cocci
			TSA-YMA	+ ; Cocci in even-numbered clusters	Small, white, raised, glistening colonies	Unknown cocci
Z023436	Sept. 4; Wadden Sea	Monoesters	TSA-TSA	+ ; Long rods, some in clusters	Raised, wrinkled, cream-colored, matte; spore-forming rods	*B. licheniformis*

[a] The order of which (sub)cultures were transferred to plates with different media.

Gas chromatography of preen waxes

Preen wax samples of all birds were obtained immediately after a feather sample or bacterial swab was taken. The wax samples were dissolved in ethyl acetate to a concentration of 1 mg ml^{-1} and injected into a gas chromatograph (Shimadzu UV-1601) using an on-column injector. Detection was accomplished using a flame-ionisation detector. Helium was the carrier gas. Separation of the chemical components was achieved using a fused-silica capillary column (Varian, 25 m x 0.32 mm i.d.) coated with CP-Sil 5CB (film thickness 0.12 μm). The samples were injected at 70°C, and the oven was subsequently heated to 130°C at 20°C min^{-1} followed by 4°C min^{-1} to 320°C, and held at this temperature for 35 min. Gas chromatograms of pure mono- or diesters are easy to distinguish and identify visually based on previous molecular analysis of the intact monoester and diester preen waxes (Dekker *et al.* 2000, Sinninghe Damsté *et al.* 2000). This enabled us to determine whether individual birds had preened either mono- or diester preen waxes onto their plumage. All birds secreted pure monoester preen waxes on 4 May, whereas the same birds secreted pure diester waxes on 17 June. The birds had not molted their breast feathers between the two sampling dates.

Treatment groups

To compare bacterial degradation of feathers coated with different preen wax compositions, feathers were collected from the 16 adult red knots. Half of the samples from each collection date were placed in ethyl acetate, a solvent of hydrophobic waxes, and gently shaken in an automatic shaker. After 8 hrs the feathers were taken out of the ethyl acetate and air-dried. Gas chromatograms of the ethyl acetate that had been used to wash the feathers showed the peak pattern typical for mono- or diester preen waxes of red knots. The ethyl acetate removed part or all of the preen waxes. We made thirty photographs with a Scanning Electron Microscope of four untreated feathers and four feathers of which preen waxes were removed with ethyl acetate. We coded the pictures to remove knowledge of whether the feathers were untreated or had the wax removed and examined them for any signs of damage (holes, broken barbules), paying special attention to where barbules connect to barbs. The ethyl acetate did not affect the feathers in any way that we could see. The washed feathers were used to measure the growth of *B. licheniformis* on feathers without waxes. In addition to looking for photographic evidence of damage, we incubated two uninoculated samples of washed feathers and one uninoculated sample of unwashed feathers to serve as controls for the effect of shaking on washed and unwashed feathers in the absence of bacteria.

Feather-degrading experiment

We followed the procedure of Goldstein *et al.* (2004) to measure bacterial degradation of feathers. Here, bacterial growth is indirectly measured by determining the concentration of oligopeptides in a medium of *B. licheniformis* with feathers. Oligopeptides are a by-product of bacterial degradation of β-keratin, the structural protein of feathers (Goldstein *et al.* 2004).

Replicates of 0.075 g feathers of each treatment group were put in 25 ml feather medium (9.34 mM NH_4Cl, 8.55 Mm NaCl, 1.72 mM K_2HPO_4, 2.92 mM KH_2PO_4, 0.49 mM $MgCl_2$-$6H_2O$ and 0.01% yeast extract) in 100 ml Erlenmeyer flasks with lids. The flasks were sterilized in an autoclave for 20 min at 15 psi and 120˚C. Gas chromatography of heated and unheated preen wax showed no difference in the structure of the different preen waxes.

After the flasks had cooled down, they were inoculated with *B. licheniformis* strain OWU 138B (available from the American Type Culture Collection as strain ATCC 55768). To prepare the inoculum, we transferred a small sample of strain 138B from an isolation tube to a 250 ml flask containing 100 ml of Luria broth and incubated the flask at 37°C and 120 rpm. After 24 h we removed 2.5 ml of bacteria and nutrient broth from the flask and placed them in 15 ml tubes. The tubes were centrifuged for 10 min at 4500 rpm to separate the nutrient broth from the bacteria. The nutrient broth was discarded and the bacteria were resuspended in 1 ml of feather medium and added to the 100 ml flasks described above.

Following inoculation the flasks were put in a 37°C incubator, rotating at 120 rpm. After 96 hrs 0.5 ml was removed from each flask and diluted with 0.5 ml of feather medium in order to obtain an adequate volume to measure the absorbance. The sample was centrifuged for 10 min at 4500 rpm to sediment the feather fragments and bacteria. The absorbance of the supernatant was measured at a wavelength of 230 nm with a Beckman DU UV/VIS spectrophotometer. At this wavelength light is maximally absorbed by the oligopeptides (Goldstein *et al.* 2004). The samples were discarded after measurement. The increase in oligopeptides leveled off after 96 hrs for some feather samples. For that reason, and because an earlier pilot study showed that the oligopeptide concentration increased linearly during the first four days, we decided to use the oligopeptide concentration 96 hrs after inoculation as our measure of feather degradation.

The initial quantity of oligopeptides in the solution had to be known in order to measure feather degradation by *B. licheniformis*; therefore, a first measurement was taken after one hr of incubation without bacteria, when the medium was well mixed, but bacteria had produced few oligopeptides. We subtracted these initial light absorbance values from those measured after 96 hrs to correct

for oligopeptides (and possible other proteins) in the feather medium that are not due to feather-degradation by the inoculum.

The data were analyzed with a repeated measures ANOVA with two within subjects ('presence of wax' and 'wax composition'). One of the four measurements was missing from three birds and these individuals were excluded from the analysis. Another individual was excluded because absorbance values were clear but unexplained outliers for all treatments (maximal absorbance of 0.0614).

Results

Occurrence of feather-degrading bacteria

Six of the seven red knots captured on the breeding grounds in Greenland had preen wax that contained only diesters and one chick-guarding bird secreted monoester preen wax. All 28 migrating red knots captured in the Wadden Sea secreted preen wax that contained only monoesters. This follows closely the pattern described by Reneerkens *et al.* (chapter 2) who showed that only incubating sandpipers secrete diester preen waxes. Bacteria found in the samples included *Streptomyces* sp., *Staphylococcus* sp., *B. licheniformis* and unidentified cocci. The feather degrading *B. licheniformis* occurred only in red knots that secreted monoester preen wax (table 6.1). This included the single, chick-guarding, bird that secreted monoester preen wax at the breeding grounds and 6 of 28 red knots during migration in the Wadden Sea. In addition, we cultured *Staphylococcus* sp. from one diester-secreting individual, and *Streptomyces* sp. and some unspecified cocci in plumages of monoester-secreting individuals during migration in the Wadden Sea. Details about the identified bacteria are given in table 6.1. The sample sizes are too small for sufficient statistical power to draw conclusions from these frequencies.

Effects of preen waxes

The rate of degradation by *B. licheniformis* of feathers with a coat of monoester waxes did not differ from that of feathers with a coat of diester waxes (repeated measures ANOVA $F_{44,1} = 0.699$, $P = 0.408$), but the removal of the wax coat from these feathers significantly increased the bacterial breakdown of the feathers (repeated measures ANOVA $F_{44,1} = 11.480$, $P = 0.001$; fig. 6.1). The interaction between 'presence of wax' and 'preen wax composition' was not significant (repeated measures ANOVA $F_{44,1} = 0.498$, $P = 0.484$). Feathers incubated in the absence of *B. licheniformis* did not degrade regardless of the presence or absence of preen wax.

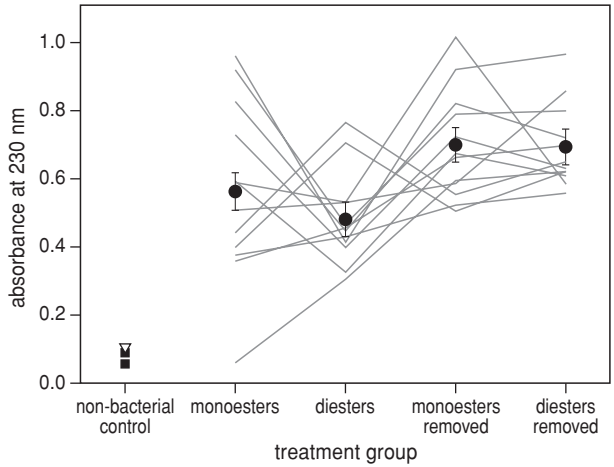


Figure 6.1 Absorption of radiation at 230 nm by media containing dissolved oligopeptides of β-keratin released after 96 h of degradation by *B. licheniformis* of red knot feathers preened with monoesters, diesters or without mono- and diester waxes, as well as the three non-bacterial controls (black squares: feathers in medium treated with ethyl acetate; white triangle: feather medium only). Symbols represent the means in accordance with the used repeated measures ANOVA (i.e. the least square means, which are the means after correction for inter-individual variation). Error bars represent standard errors of these means. Grey lines connect the absorbance measurements of each individual.

Discussion

Here we show for the first time that red knots harbor feather-degrading bacilli in their plumage during incubation on the High Arctic breeding grounds and at intertidal migration stopover sites in temperate climates. This is the first evidence that *B. licheniformis* occurs in sandpipers (Scolopacidae). Its occurrence supports the conclusion of Burtt & Ichida (1999) based on the pattern of occurrence in passerines, that *B. licheniformis* would be found in the plumage of all avian taxa. Sample sizes were too small to draw definite conclusions about the differential occurrence of *B. licheniformis* in plumages of breeding red knots that secrete diester preen waxes and non-breeding individuals that secreted monoesters, although it is striking that *B. licheniformis* only occurred in monoester secreting red knots. Future study of seasonal changes in presence of feather bacteria is needed.

This is the first time that growth inhibition of feather-degrading bacteria has been tested with feathers to which preen waxes were applied by the birds themselves. We show that preen waxes in the amounts preened onto the feathers by

red knots effectively diminish feather-degradation. These results are consistent with those of disc-diffusion experiments (Shawkey *et al.* 2003) that showed that preen wax of house finches *Carpodacus mexicanus* delayed the growth of *B. licheniformis*.

It remains to be investigated whether *B. licheniformis* is able to degrade feathers under natural conditions on living birds. Cristol *et al.* (2005) could not detect feather damage caused by experimentally applied bacteria on plumages of captive songbirds. However, they could not exclude the possibility that no feather damage was found due to preening, sunning (Saranathan & Burtt 2007), or other maintenance behavior of the birds. They also argued that the optimal growing conditions for *B. licheniformis* (temperatures around 45 ºC, humid conditions) do not often occur under natural circumstances (Cristol *et al.* 2005). Although the temperatures in clutches incubated by High Arctic breeding shorebirds are approximately 36 ºC (Cresswell *et al.* 2004), the temperature of the plumage in these conditions is probably higher and may approach the optimal temperature for *B. licheniformis*. Additionally, the microclimate in bird nests is relatively humid (Ar & Sidis 2002). However, diester preen waxes secreted during incubation, when the damp, warm environment of the nest scrape may favor bacterial growth, did not protect of the plumage from potential bacterial degradation better than the usually secreted monoesters.

If we want to understand inter- and intraspecific variation in preen wax composition in the light of co-evolution with microbes on birds' plumage (Shawkey *et al.* 2003, Sweeney *et al.* 2004), the mechanisms responsible for the inhibition or enhancement of microbial growth by preen waxes need to be understood. How would preen waxes inhibit bacterial growth on feathers? Shawkey *et al.* (2003) suggested that preen waxes act as a chemical repellent in which alkyl-substituted fatty acids and alcohols are anti-microbial agents. Indeed, Jacob *et al.* (1997) showed that 3,7-dimethyloctan-1-ol, one of the products of hydrolysis of preen wax of gannets *Morus bassanus*, negatively affects growth of Gram-positive bacteria and dermatophytes. However, preen waxes of most bird species consist of esters, which are fatty acids condensed to alcohols, but free fatty acids or alcohols rarely occur in preen wax secretions (Jacob 1976, Jacob & Ziswiler 1982, Dekker *et al.* 1999, Sweeney *et al.* 2004), and not even in the preen waxes of gannets (Jacob *et al.* 1997). It remains to be seen whether hydrolysis of preen waxes takes place under natural conditions, e.g. under the influence of ultraviolet light or by bacteria that use waxes as a substrate.

Our study suggests that the chemical composition of the wax esters does not affect their anti-bacterial capacities. Preen gland secretions consist of complex mixtures of often more than one hundred different types of wax esters that vary in chain length and branching (Jacob & Ziswiler 1982, Haribal *et al.* 2005). The

Incubating red knots create a relatively warm and humid microclimate in the nestcup that is likely also favourable for the growth of *Bacillus licheniformis*.

chemical composition of the preen wax mixtures affects their physical characteristics (e.g., melting temperatures, Patel *et al.* 2001). However, all avian preen waxes consist of chemically stable esters. Therefore, we propose that preen waxes do not chemically combat microbes, but form a physical barrier between microbes and feathers.

More knowledge of the physical aspects of preen wax esters as well as on the (micro-) distribution of preen waxes on the plumage will be required to test this idea. Although diesters are larger molecules than monoesters (Sinninghe Damsté *et al.* 2000), which should affect mechanical properties, the different preen wax mixtures found in red knots did not differ in their ability to inhibit growth of feather-degrading bacteria. Future descriptive and experimental studies of the function of inter- and intraspecific variation in preen waxes in an ecological context need to consider the chemical and physical aspects of the secretions. Such studies should not only focus on the interaction between preen wax secretions and microbial flora (Shawkey *et al.* 2003) or ectoparasites (Moyer *et al.* 2003), but should also consider other selective factors, such as mate choice and predation (cf. chapters 5,6 and 7) and also include (seasonal) quantitative variation in preen wax secretion (Bhattacharyya & Roy Chowdhury 1995, Montalti & Salibián 2000).

Acknowledgements
We would like to thank Laura Tuhela-Reuning, David Lever and Jerry Goldstein at Ohio Wesleyan University and the Microbiological Ecology Group, especially Henk Bolhuis, at Groningen University for their help and the use of their laboratory. The work was supported by ALW grant 810.34.003 to Theunis Piersma and Jaap Sinninghe Damsté of the Netherlands Organisation for Scientific Research (NWO) and a National Science Foundation grant (BIR-998805) to Edward H. Burtt, Jr. Maaike A. Versteegh was supported by a student grant of the Marco Polo Trust. Amy M. Schneider was supported by a National Science Foundation Summer Research Fellowship (NSF BIR-998805). Our expedition to Greenland received financial support from the Netherlands Arctic Programme, administered by the Netherlands Organisation for Scientific Research (NWO). Joop Jukema, Petra de Goeij, Welmoed Ekster and Hans Meltofte contributed to the pleasure and success of the field work and the Danish Polar Center is acknowledged for excellent logistical support in Greenland.

Box D The effect of preen wax on the abrasion-resistance of primary feathers: a field experiment on High Arctic breeding sandpipers

Jeroen Reneerkens & Theunis Piersma

Most birds possess a preen, or uropygial, gland from which lipid secretions (preen waxes) are smeared with the bill onto the feathers during preening activities (Jacob & Ziswiler 1982). Despite the ubiquity of the preen gland among birds, it still is largely unclear which function(s) the secreted waxes serve. The available experimental tests indicate that presence of preen wax reduces growth of feather-degrading bacteria (Shawkey *et al.* 2003, chapter 6), repels feather lice (Moyer *et al.* 2003) and that seasonal changes in preen wax composition cause a temporal (relative) olfactory crypsis against mammalian predators that use smell to locate prey (chapter 7). In some bird species, the preen gland secretions enhance colouration of the plumage (Delhey *et al.* in press), but not in others (chapter 5). Many more functions of preen wax have been proposed in the past but, to our knowledge, experimental evidence for these functions are lacking.

In the old discussion about the possible functions of preen waxes, an often proposed function is the protection of feathers against tear and wear (e.g. Elder 1954, Jacob & Ziswiler 1982). Feathers may wear by ultraviolet radiation (Bergmann 1982) and by contact with hard objects such as vegetation and airborne particles (Burtt & Ichida 2006) and by degradation by ectoparasites (Clayton 1990). That feather wear can have substantial effects

that accumulate over time becomes obvious when we consider that in great snipes *Gallinago media* individuals in their first summer plumage, that renew their primary feathers only in the next summer, can be distinguished from adults in summer plumage, that have primary feathers that are created in the past autumn, by the wear of their primary wing tips (Saether *et al.* 1994). In coastal shorebirds, in the middle of the non-breeding season, juveniles (that have carried their primaries for longer than adults) generally can be distinguished on the basis of primary wear (Prater *et al.* 1977).

It has been shown that different kind of feather keratins differently affect abrasion resistance, in which melanised feathers are stronger and resist tear and wear and bacterial degradation better than unmelanised feathers (Burtt 1981, 1986, Goldstein *et al.* 2004). Although recent studies show that preen wax protects feathers againsts ectoparasites that may cause feather degradation (Bandyopadhyay & Bhattacharyya 1996, 1999, Moyer *et al.* 2003, Shawkey *et al.* 2003, chapter 6), the effects of preen wax against physical wear and tear have remained unexplored. Rutschke (1960) suggested that preen waxes penetrate into the medulla cells of the barbs and shafts of feathers and thereby increase the flexibility of feathers that thereby break less easily. This effect and the penetration of preen wax into feathers itself have, however, never been substantiated. Here we examine the role of preen waxes secreted by sandpipers during incubation (consisting of diesters only, chapter 2) on the abrasion resistance of feathers in a relevant field context.

Methods

During the arctic summer of 2003 we tested this hypothesis in Zackenberg, Northeast Greenland (74°30'N, 20°30' W). From 17 June till 15 July 2003 we searched for nests of dunlins *Calidris alpina arctica*, ruddy turnstones *Arenaria interpres*, sanderlings *Calidris alba*, red knots *Calidris canutus islandica* in a tundra area of more than 4 km^2 in the vicinity of the research station (Piersma *et al.* 2006). During the incubation period we tried to catch both of the incubating adults from the nest by use of small clap-nets. A small smear from the preen gland for chemical analysis in the laboratory of the Royal NIOZ was collected following procedures described by Reneerkens *et al.* (chapter 2).

Field experiment

We cut off 1 cm long tips of both the left and right 8th primary (P8; wing feather) with sharp scissors of the caught birds and collected the feather tips into small glass vials. The vials containing the feather tips were stored at 5 °C and kept still to avoid any possible damage to the feathers by scratching against the sides of the vial. The feather tips of the P8's served as a control for possible *a priori* differences in abrasion between the two wings that were not related to the experimental treatment. The treatment consisted of chemically removing preen wax from a randomly chosen (left or right) wing by dissolving the wax into ethyl acetate, a potent solvent of hydrophobic waxes. This was done by stirring around the wing tip in a glass vial filled with ethyl acetate for *ca.* 30 sec, after the P8 feater tips were collected. The ethyl acetate, which is volatile at normal ambient temperatures, was allowed to evaporate from the wing in the field after which the birds were released again. The individual birds were recaptured on their nests after 8 days on average (range 1–28 days). At recapture, feather tips of the ninth primary (P9) were collected following the same procedure as for the P8 earlier. The ninth primary tips were collected to look for a treatment effect of preen wax removal. Our null-hypothesis was that removing preen wax would not result in different abrasion of the wing feathers during the days between preen wax removal and recapture. The left and right wings could be compared within an individual as preen wax was removed from only one of the two wings. In total 28 birds were treated and recaptured; nine dunlins, one red knot, three ruddy turnstones, and fourteen sanderlings. An additional twenty-seven birds were treated but could not be recaptured for the collection of the P9's.

The feather tips were studied with a dissection microscope (magnification 20–40) and scored for abrasion. The first 15 barbs starting from the tip of the feather both from the inner and the outer vane were given an abrasion score between 0 and 5. A score of 0 was an intact non-abraded barb, 1: a tiny tip of the barb was worn, 2: a small tip of the barb was broken off, 3: a significant part of barb missing, 4: up to half of the barb missing, 5: more than half of the barb missing. The scores of the 15 barbs from the inner and outer vane were added up to a 'total abrasion score'.

The total abrasion scores were not normally distributed. Hence, we used a Wilcoxon paired-sample test to test for *a priori* differences in total abrasion scores of the P8 on the side to be treated and the side that was not going to be treated with ethyl acetate, and similarly for treatment effects on the P9's.

Results

Total abrasion scores ranged between 6 and 135 (fig. D1). The control feather tips (P8) of left and right were not different from each other (Wilcoxon Signed Rank test, $P = 0.484$, fig. D2). Abrasion scores of the P9 with or without temporary wax removal also did not differ (Wilcoxon Signed Rank test, $P = 0.484$). As expected, given its position more to the end of the wing, tips of P9 were abraded more than the tips of P8 (Wilcoxon Signed Rank test, $P < 0.001$).

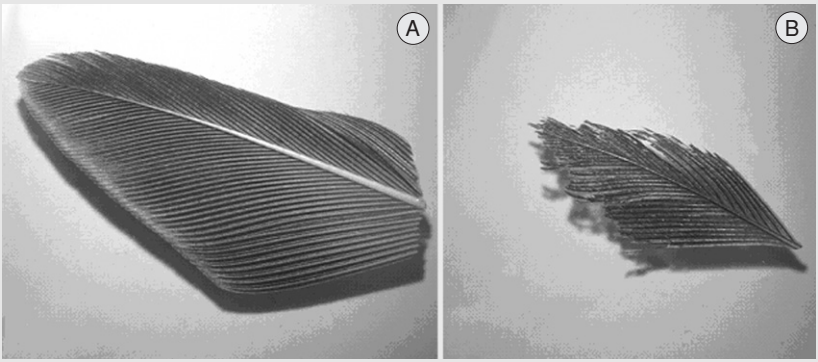


Figure D1 An example of two feather tips of sanderlings with the most extreme abrasion scores encountered. The feather tip in (A) had a total abrasion score of 6 and a score of 135 in (B). The used microscope magnification for both pictures is 40.

Discussion

The removal of preen waxes from the wing tip did not result in a significantly different abrasion within the 1- 28 days that the experiment lasted. There are several possible explanations for the lack of an effect of chemical preen wax removal on primary wing feather abrasion. First of all, the occurrence of preen wax on feathers might not play a biological role in the protection of feathers against tear and wear. It can, however, not be excluded that the birds had preened wax onto their treated wing, soon after we had experimentally removed the wax. In any case, however, (part of) the preen wax will have been temporally absent from the wing feathers. It is also possible that the experiment lasted too shortly for significant abrasion in feathers to take place.

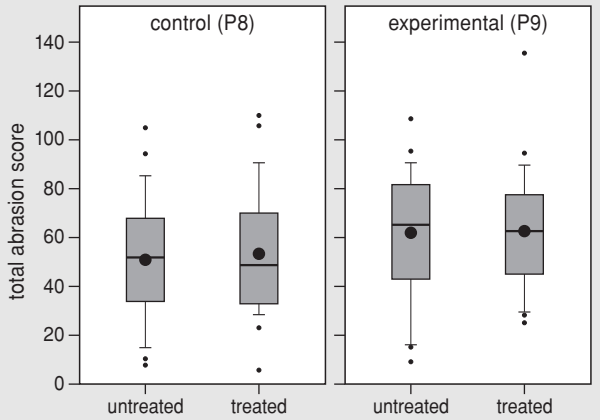
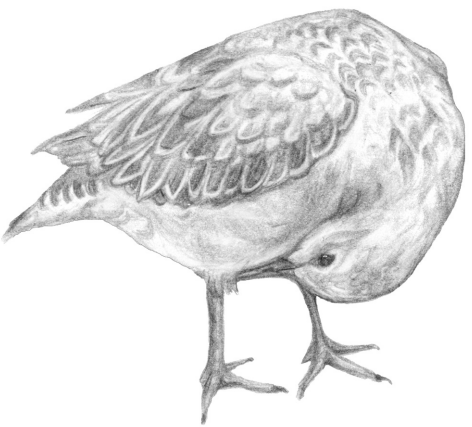


Figure D2 Total abrasion scores of the eight (P8) and ninth primaries (P9) of 28 shorebirds. A distinction is made between the feather tips that were treated with ethyl acetate to remove preen waxes and those that were not treated. Note that the P8's served as a control and both sides were never treated with ethyl acetate. The boxes enclose 50% and vertical lines 95% of the value. The small dots are outliers. Black dots represent the average values, the dividing lines the median.

The different abrasion scores between P8 an P9 suggest that outer primaries are more subjected to wear than more inner wing feathers. This can possibly be explained by the fact that outer primaries are more exposed and cover larger distances with each wing movement. This aspect deserves further attention, as it may be interesting with respect to the evolution of moulting strategies and/or preening behaviour.

Acknowledgements
We would have had to conduct this study with much lower sample sizes if we did not have had the help of Welmoed Ekster, Petra de Goeij and Joop Jukema who spent many long days with us on the tundra near Zackenberg searching for sandpiper nests. The excellent logistical support by the Danish Polar Centre and good company in Zackenberg is greatly appreciated. We thank the Netherlands Arctic Programme, administered by the Netherlands Organisation for Scientific Research (NWO) for financially supporting our work in Zackenberg in 2003.



CHAPTER 7

Switch to diester preen waxes may reduce avian nest predation by mammalian predators using olfactory cues

Jeroen Reneerkens, Theunis Piersma & Jaap S. Sinninghe Damsté

Journal of Experimental Biology 208: 4199-4202

ABSTRACT

It has long been recognised that nest depredation by olfactory-searching mammals greatly influences the reproductive success of ground-nesting birds. Yet adaptations of birds to diminish smell during nesting have rarely been investigated. Recently, a remarkable shift in the composition of uropygial gland secretions (preen waxes) was discovered in many ground-nesting shorebirds and ducks that begin incubation, during which the usual mixtures of monoester preen waxes are replaced by mixtures of less volatile diester waxes. In this study we show experimentally that an olfactory-searching dog had greater difficulty detecting mixtures of the less volatile diesters than mixtures of monoesters. This is consistent with the hypothesis that diester preen waxes reduce birds' smell and thereby reduce predation risk.

Introduction

The application of secretions of the uropygial gland, also called preen waxes, is an important aspect of plumage maintenance in birds. Preen waxes repel water (Jacob & Ziswiler 1982) and inhibit the growth of feather-degrading bacteria (Shawkey *et al.* 2003). In the European oystercatcher *Haematopus ostralegus*, six plover species (Charadriidae), and at least 19 sandpiper species, including the red knot *Calidris canutus* (chapter 2 and 7), preen wax composition changes over an annual cycle from lower molecular- mass monoester waxes (total carbon number distribution in the range C_{24}–C_{26} and C_{30}–C_{38}) to higher molecular-mass diester waxes (total carbon number distribution in the range C_{32}–C_{48}; Sinninghe Damsté *et al.* 2000). The shift to diester preen waxes is completed when the birds are ready for a long northward flight to High Arctic breeding grounds (chapter 2), where courtship starts soon after arrival.

Red knots that departed on, or arrived after, the first part of a non-stop migratory flight of several thousand km did not secrete diester preen waxes. Birds of the same population, ready for the second part of the migratory journey to the High Arctic breeding grounds, did, however. Therefore, it was suggested that secretion of diester preen waxes was not related to long-distance flights *per se* but that the timing of the compositional preen wax shift was apparently related to breeding activities (Piersma *et al.* 1999).

Our first hypothesis, that diester waxes enhance plumage colouration and function as an individual quality signal during courtship (Piersma *et al.* 1999), is not the only explanation for the observed shift in preen wax composition, as spectral measurements of plumages of red knots before and after the shift to diester preen waxes showed no difference in colouration (chapter 5). Furthermore, the secretion of diester preen waxes continues during incubation, with a return to monoesters when the chicks hatch. A similar shift to diester preen waxes during incubation has already been found in wild-type and domesticated mallards *Anas platyrhynchos* (Jacob *et al.* 1979, Kolattukudy *et al.* 1987), which are also ground-breeders. For species whose males do not incubate, the shift to diester preen waxes is limited to the incubating females (chapter 3). This indicates that the diester wax cocktail fulfils a specific function during incubation, but that the function during this crucial phase is unknown (chapter 2). Ground-nesting birds are particularly vulnerable to loss of their clutch to predators (Whelan *et al.* 1994), which can greatly influence the population dynamics of ground-nesting birds (Blomqvist *et al.* 2002). Because of their high molecular mass, diesters are less volatile than monoesters and might thus be more difficult to detect by olfactory-searching predators. In this study we tested this hypothesis using a sniffer dog trained to locate different amounts of pure mono- or diester preen waxes.

Materials and methods

Preen waxes

We collected pure preen wax biweekly from 14 red knots *Calidris canutus* kept in outdoor aviaries from 1 March 2002 to 24 June 2002 by softly massaging the papilla of the gland with a cotton bud. Waxes were extracted with ethyl acetate, weighed and dissolved in ethyl acetate (1 mg wax ml^{-1}). The solution was injected into a gas chromatograph (Hewlett-Packard 6890 Series II) using an on-column injector. Detection was accomplished using a flame-ionisation detector. Helium was the carrier gas. Separation of the chemical components was achieved using a fused-silica capillary column (Varian; 25 m x 0.32 mm i.d.) coated with CP-Sil 5CB (film thickness 0.12 μm). The samples were injected at 70°C, and the oven was subsequently heated to 130°C at 20°C min^{-1} followed by 4°C min^{-1} to 320°C, and held at this temperature for 35 min. From previous detailed molecular analysis of the intact monoester and diester preen waxes we learned that their gas chromatograms are characteristic for either monoester or diester preen waxes (see inset in fig. 7.1A; Dekker *et al.* 2000, Sinninghe Damsté *et al.* 2000). For the purpose of this study, this enabled us to examine the gas chromatograms visually to determine which preen wax composition (mono- or diesters) was secreted at a given date. To characterise the relative abundance of mono- and diesters in the preen wax of an individual bird at a given date (fig. 7.1A), appropriate peak areas in the gas chromatograms were integrated. The percentage of diesters in the wax mixture was estimated following the formula % diester = surface diester peaks / (surface diester peaks + surface monoester peaks) x 100. This relative abundance of diesters was averaged for all individual birds on a given day and the 95% confidence intervals, as shown in fig. 7.1A, were calculated.

On 23 April 2002 all captive birds secreted pure monoesters whereas on 11 June 2002 only diesters were produced. We combined the 14 samples from each of these days (8.4 and 7.9 mg waxes, respectively) and dissolved them in ethyl acetate to an exact concentration of 1.0 mg wax ml^{-1}. These two samples were the basis for serial dilutions of preen wax with ethyl acetate. At each step the concentration was reduced by a factor of two.

Under a fume hood we pipetted 0.5 ml of the solution to square metal rods that were lying on clean aluminium foil. The solution was equally spread over two sides of the square rods using a pipet, such that the side with waxes never touched the aluminium foil. Control rods were applied with 0.5 ml pure ethyl acetate. After evaporating off the volatile solvent, the metal rods were kept in airtight glass jars. Rods and glass jars were boiled in water for 10 min and washed without detergent in a dishwashing machine before use. Metal rods and glass jars were never touched and always handled using a pair of metal pliers.

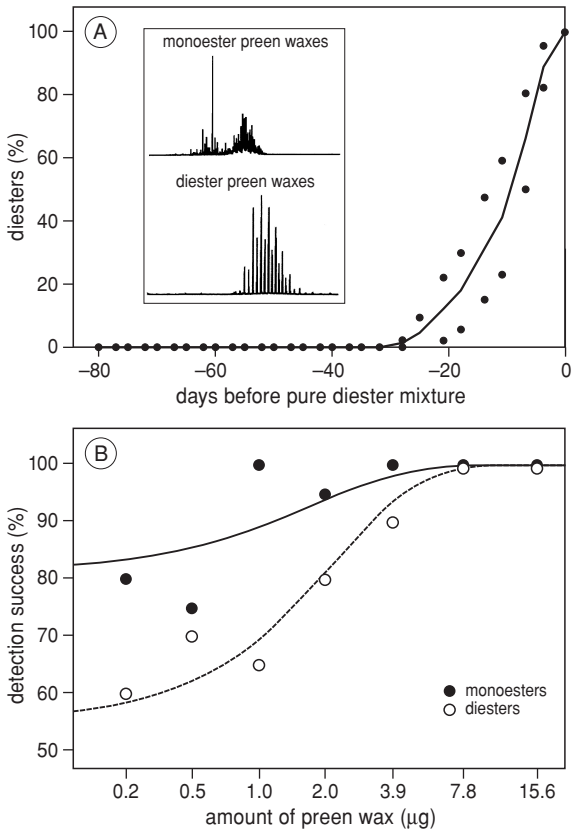


Figure 7.1 Red knots shift from mono- to diester preen waxes, the latter being more difficult to detect by a sniffer dog. (A) The shift from mono- to diester preen waxes (see inset) in spring takes place within 1 month in individual captive red knots (N=14 individuals; 95% confidence intervals around mean values of percentage of diesters are indicated by dots). (B) The likelihood of successful detection is a function of the type and amount of preen wax. Each data point represents detection success during 20 sessions (monoesters: black circles, diesters: open circles). Fits from the used logistic model (see Materials and methods) are depicted in the graph as lines (solid, monoesters: ln(Pdetection/1–Pdetection) = 1.4519+0.6602 x amount; broken, diesters: ln(Pdetection/1-Pdetection) = 0.1786+0.6602 x amount).

Sniffer dog

We trained a 6-year-old female German shepherd dog to locate different amounts of both mono- and diester waxes. The dog had positive health certificates on stamina and had been recommended for breeding. Initially, the dog was taught to sniff systematically a row of six plastic tubes mounted 1 m apart on a wooden

Sniffer dog Joey in action during the experiment. A short movie clip of the experiment can be found at http://jeb.biologists.org/cgi/content/full/208/22/4199/DCI.

board, to locate the metal rod applied with smell of the dogs owner at a randomly chosen position and be rewarded for it by being allowed to play with the rod for some time and by compliments from the trainer. The dog trainer applied his own smell to the rod by touching it and keeping it in his pocket. Control rods remained untouched and were placed in the remaining locations. After the dog had located the rod with the smell of the dog trainer convincingly several times, human smell was replaced by 1 mg mono- or diester preen waxes. To get an idea of the amounts at which the dog started to fail locating the preen waxes, the amounts of preen waxes on the rod were gradually decreased during the training procedure. Training took place from January 2003 to February 2004, and the ac-

tual experiments on four different days during the period February to July 2004 in familiar surroundings, in the garage of the dog-owner.

The experiment was performed with different amounts of mono- and diester preen waxes, between 0.24 and 15.6 μg. The intention of the experiment was to examine whether detection probabilities are equal for the same amounts of preen wax. Under natural conditions, the quantity of wax molecules in the air will depend on the distance from the source. The amounts of preen wax used in this qualitative experiment, in which the dog sniffed the rods at a distance of only a few cm, therefore do not need to reflect the natural amounts expressed by birds. The order of sessions with respect to composition (mono- or diesters) and amount of preen wax was randomised. Dog and trainer were unaware of the location of the treated rod. If it had smelled the preen wax, the dog would take the metal rod. On failing to locate the rod with wax, the dog continued systematically searching the row of tubes, sometimes up to 30 times before giving up. On giving up, the dog often started searching elsewhere in the room where the experiments were carried out. The dog never indicated a finding of preen wax on control rods, i.e. never made a mistake.

The success with which the dog located the wax was scored for wax composition and amount. Each combination of wax composition and amount was tested 20 times (280 experiments in total) over 4 days, on each of which all combinations of wax amounts and composition were tested five times. Detection chance ($P_{detection}$) was analysed using a logistic model $\ln(P_{detection}/1\text{-}P_{detection}) = a+b \times \text{amount}$, which was fitted to the data by iteration (Crawley, 1993). Factors (amount of wax, wax composition and their interaction) were added separately to the model and a χ^2 test was used to estimate whether the addition of factors caused significant reductions of deviance.

Results

The complete shift from mono- to diesters in the preen wax composition of individual red knots took place within a month (fig. 7.1A). Diesters are less volatile than monoesters, as indicated by their gas chromatograms (inset in fig. 7.1A). With decreasing amounts of preen wax the dog increasingly failed to locate them (fig. 7.1B). The model that included both main factors (amount and composition of preen wax) significantly contributed to the fit compared with models that included only a single main factor (from the model with amount as a factor only: $\chi^2 = 10.5$, d.f.$=1$, $P<0.005$; from the model with composition only: $\chi^2 = 40.1$, d.f.$=1$, $P<0.001$). This tells us that the decline in detection success with lowering amounts of preen waxes is steeper for diester waxes than for monoester waxes.

Discussion

The results of our experiment using the single sniffer dog are consistent with the idea that diesters are more difficult to smell than monoesters. This suggests that the use of diesters during incubation would improve 'olfactory crypsis'. Although it is unknown how much preen wax is expressed by incubating birds, the smell of preen waxes, and hence detection chance, will decrease with distance from the bird. At a certain distance from the source the smell of preen wax will reach critical levels at which predators might not detect them. The results of our experiment suggest that this maximum detection distance is smaller for diester than for monoester preen waxes.

Predation of eggs by mammalian, olfactory-searching predators largely determines the reproductive output of young sandpipers (Blomqvist *et al.* 2002). This severe natural selection has led to the evolution of cryptic plumage and egg coloration to conceal nests and incubating birds from visually searching egg predators (Solís & De Lope 1995, Jukema *et al.* 2003a). It has long been known that many mammalian predators rely on smell to locate prey (Whelan *et al.* 1994), and although folk wisdom relates that snipe and quail are impossible to detect by hunting dogs as long as they are on their nest (box E) to the best of our knowledge, this is the first experimental test of olfactory crypsis as a potential complementary anti-predation strategy. Future research should reveal if our findings using a single dog can be generalised to natural predators in a field situation where detection probabilities also depend on distance from the incubating bird, wind conditions and habitat characteristics.

Seasonal shifts in preen wax composition are presumably the result of a changing balance between costs and benefits of the production and use of diester rather than monoester preen waxes. In the non-breeding season sandpipers live in large flocks and can fly away from mammalian predators. Their reliance on monoesters during times when olfactory crypsis is unimportant suggests that the production or use of diesters carries a cost. A greater understanding of the energetic costs and of functional properties, such as anti-parasitic aspects or waterproofing, of mono- and diester preen waxes is necessary to better understand seasonal shifts in preen wax composition.

Acknowledgements
This experiment would not have been possible without the intensive help of Ton van der Heide, who took care of the dog training. Adee Schoon gave useful advice on dog training. Jaap van der Meer helped with the statistical analysis. Bird handling was carried out under the auspices of the Animal Experiment Committee (DEC) of the Dutch Royal Academy of Sciences (KNAW). Our work is financially supported by ALW grant 810.34.003 of the Netherlands Organisation for Scientific Research (NWO) to T.P. and J.S.S.D.

Box E Scent of a quail: toe cheese or bad breath?

From: http://teamquail.tamu.edu/v1n3.htm

Perhaps I should ask this of Al Pacino. What is the origin of a quail's scent? In other words, physiologically speaking, what allows my setter Suzie to detect the presence of quail, peaking ultimately in a stylish point?

From what I've read in the literature, the scent is produced by gases produced by bacteria growing on the epithelial cells of a quail's foot. As the bird moves around, cells are sloughed, bacterial growth occurs, and scent ensues. But I have a hard time with that explanation.

Consider this. Can a dog detect a quail's scent in cold weather, when one would expect bacterial growth to be nigh? Yes.

Maybe it's the bird's uropygial (oil) gland. What is the composition of that bottled "quail scent" that you see in the sporting goods stores? Does it work? Or maybe the birds produce some type of excretion that we're not onto yet.

Or maybe it's something to do with the bird's breath. That's my conjecture. Will your dog typically point a dead quail? Mine won't. It will find one, but won't point it. But a wounded bird (thus still respiring) is treated as a live bird (as indeed it is) and pointed.

And here's another enigma. Has your dog ever pointed a quail hen (or rooster) with the bird sitting on a nest? Mine never have, and I work them year-round. Now, it makes sense that an incubating quail minimizes its scent, but how? If the incubating bird's respiration slows, it would support my theory of bad breath. Ideas or observations?

CHAPTER 8

General discussion: Identifying the adaptive value of variation in preen wax composition: approaches for future studies

Jeroen Reneerkens

In this thesis, the seasonal variation in preen wax composition in sandpipers (Scolopacidae) has been studied. In this final chapter the main conclusions are summarised, our findings are placed in the light of the current understanding of functions and evolution of variation in preen wax composition and suggestions for future research are made.

Key findings of this thesis

The main motive for the present study were the findings by Piersma *et al.* (1999) that preen wax composition in sandpipers changes shortly before the start of the breeding season from mixtures consisting of monoesters to mixtures consisting of diester waxes only. The monoester and diester preen wax mixtures were chemically described in detail (Dekker *et al.* 2000; Sinninghe Damsté *et al.* 2000) after which a nice opportunity arose to study intraspecific variation in preen wax composition in a biological context. Diesters and monoesters have a similar chemistry; both are mixtures of esters. Diesters are generally larger molecules that are more viscous and less volatile. Based both on the timing of the shift and on the physical aspects of monoester and diester waxes, Piersma *et al.* (1999) proposed that the shift in preen wax composition from mono- to diesters may serve as an individual quality signal; a 'cosmetic' produced for a short time during mate choice. This hypothesis was primarily based on the timing of the shift that suggested a quality signalling function during mate choice, as well as the exclusion of unlikely explanations. As the shift to diester preen waxes is a shift to less volatile components, instead of vice versa, a function as pheromones was rendered unlikely. The increased viscosity of diesters compared with monoesters would be a 'handicap' securing the honesty of the presumed sexually selected trait (cf. Zahavi 1975, Piersma *et al.* 1999).

In this thesis, the hypothesis of Piersma *et al.* (1999), as well as other hypotheses that may explain the seasonally changing preen wax secretions of red knots, are investigated. The 'avian make-up' hypothesis implies two testable assumptions, that are both addressed: (1) the production or application of diester preen waxes entail higher costs to individual red knots than monoester preen waxes, and therefore their use should be limited to the period of mate choice when cosmetics are presumed to be functional only and (2) the shift from mono- to diester preen waxes onto the plumage of red knots brings about a change in plumage appearance that is visible to conspecifics.

The higher viscosity of the diester preen waxes, especially in the prevailing low temperatures of the High Arctic breeding grounds of red knots, is presumed to cause diester waxes to be more difficult to apply during preening than the usual monoesters, and can be considered a 'time cost' that guarantees the hon-

esty of the signal (Piersma *et al.* 1999). Observations on captive red knots showed that they spent only 2.9% of all preening bouts (7.2% of the daily time budget) on applying preen waxes onto the feathers (Box A), a figure comparable with the 3.1% of preening bouts involving wax application observed for Swallows (Møller 1991). Even if the application of diesters would entail a doubling of time compared with monoesters, it would still not entail high costs to birds. What remains is the possibility that the cost is not a time cost, but an interaction between preening ability and wax type that may make some individuals look particularly good, but not others.

A comparative study on preen wax composition in 19 sandpiper species (chapter 2) revealed that secretion of diester waxes extended beyond the prebreeding period of mate choice. Diester preen waxes were also secreted during the period of incubation (chapter 2), and more importantly, only by the incubating sex (chapter 3). This strongly suggests that diester preen waxes are particularly important for birds during incubation. That in a sandpiper species such as the ruff *Philomachus pugnax*, in which males invest a lot in sexually selected plumage and behavioural traits (van Rhijn 1991, Lank & Dale 2001), it is the less conspicuous females that secrete diester preen wax during the breeding season rather than the male (chapters 2 and 3) suggests that selection pressures during incubation prevail over those during mate choice.

When the latter possibility was tested using spectrophotometry, the shift in preen wax composition did not appear to change the colouration of red knots' plumages, also not in the ultraviolet part of the light spectrum that is visible to birds but not to humans (chapter 5). Although the wax collected from the preen gland looks yellowish to human investigators, and although diester waxes absorb more ultraviolet light than monoesters, the removal of preen wax from the breast feathers of red knots did not affect plumage reflection (chapter 5). Possibly, the amount of preen wax on the plumage is too small to play a role in colouration. This finding also excludes the hypothesis that a decrease in (ultraviolet) light reflectance with a shift to diester preen waxes, makes incubating birds less conspicuous to (aerial) predators (cf. Viitala *et al.* 1995), and that birds would be selected on the basis of this trait during the period of mate choice (chapter 2).

The question why red knots and other sandpiper species nevertheless secrete diester preen waxes already in the period that precedes incubation, during mate choice, is discussed in chapter 4. The shift from mono- to diester preen waxes in wild and captive red knots takes at least two weeks, up to a month. Apparently, birds are not able to change preen wax composition rapidly. An endogenously controlled annual rhythm in preen wax composition would ensure that the birds start preen wax shifts in time and that the animals are fully prepared for incubation (chapter 3 and 4).

Diester preen waxes in sandpipers thus appear to have no cosmetic or otherwise visual function and are especially associated with the incidence of incubation: the hypothesis of Piersma *et al.* (1999) is falsified in more than one way. An alternative functional explanation for the seasonal changes in preen wax composition in sandpipers from mono- to diester preen waxes were thus sought in ecological circumstances during incubation. The Arctic conditions (low ambient temperatures, high wind speeds, 24 hrs daylight etc), that most sandpipers experience during incubation, presumably played no role in shaping seasonal changes in preen wax composition because several bird species that breed in temperate regions, such as several sandpipers (chapter 2), oystercatchers *Ostralegus haematopus* (Reneerkens *et al.* 2006), as well as female mallards *Anas platyrhynchos* (Jacob *et al.* 1979, Kolattukudy *et al.* 1987) secrete monoesters during most of the year and diesters during incubation as well. In this thesis two additional possible selective forces that may have lead to the evolution of seasonal shifts in preen wax composition in sandpipers were experimentally investigated: (1) feather-degrading bacteria, and (2) mammalian, olfactory-searching predators.

The plumage of birds harbours a variety of bacteria, many of which are able to degrade feathers, such as *Bacillus licheniformis*, a common feather-degrading bacterium amongst birds (Burtt & Ichida 1999, 2004) and may thus negatively affect birds. Cristol *et al.* (2005) could, however, not find effects of experimentally applied feather-degrading bacteria on plumages of captive passerines and concluded that this may be caused by the absence of warm, moist environments in which vegetative cells of feather-degrading bacteria become metabolically active and degrade feathers rapidly (Burtt & Ichida 1999). The conditions in nest cups of incubating shorebirds are relatively warm (*ca.* 36°C for an Arctic shorebird, Creswell *et al.* 2004) and moist (Ar & Sidis 2002). Therefore, incubating shorebirds are more likely to encounter metabolically active feather-degrading bacteria than non-incubating shorebirds. It was hypothesised that diester preen waxes provide a better protection against these bacteria than monoester waxes. The fact that feathers of which mono- or diester preen wax was removed were degraded at a higher rate (chapter 6), confirmed the results of previous studies (e.g. Shawkey *et al.* 2003) that preen wax does inhibit growth of *Bacillus licheniformis*. We could, however, not find a different rate of feather degradation by *Bacillus licheniformis* in media of breast feathers of red knots with natural amounts of monoester waxes and diester waxes (chapter 6).

Sandpipers are ground-breeders. Hence, their clutches are not protected against egg predators by physical barriers. In the open, sparsely vegetated habitat of the High Arctic, where sandpipers such as sanderlings *Calidris alba* and red knots *Calidris canutus* breed, it is also impossible for birds to hide their nests in vegetation. Their cryptic plumage and silent behaviour reduce detection proba-

bilities of the incubating birds by predators (van de Kam *et al.* 2004) and researchers alike (Piersma *et al.* 2006). The most common predators of shorebird eggs are mammals with an unusually well-developed sense of smell, such as Arctic foxes *Alopex lagopus*. Therefore, visual camouflage only is not sufficient to diminish or avoid detection by such predators. With a trained sniffer dog it was experimentally examined whether the less volatile diester preen waxes that are secreted during incubation, are more difficult to detect by a mammalian predator than the usual monoester waxes. This appeared to be the case. If everything else is equal and if Arctic foxes have a sense of smell that is similar to dogs, this suggests that incubating sandpipers that apply monoesters onto their plumage can be detected by olfactory-searching predators from a larger distance, and thus have a larger chance of losing their clutch to a predator, than sandpipers that secrete diesters when nesting (chapter 7). Predation of clutches in rather short-lived sandpipers that usually lay only a single clutch per year is a strong selection pressure. Adaptations that result in a smaller predation risk will presumably be strongly selectively favoured and we believe that the reduction of predation risk by the application of diesters rather than monoesters has lead to the use of diester preen waxes in incubating birds.

Seasonal variation in preen wax composition is likely the outcome of seasonally changing balances between costs and benefits of chemically different preen wax secretions. Natural selection is expected to select against costly traits if there are no benefits involved that outweigh the costs of such traits. The benefits of the use of diesters during incubation are presumably a reduction of egg predation. The costs associated with the use of diesters, relative to monoesters, still need to be established and have not been found in this thesis.

Understanding variation in preen wax composition

The scientific debate about the function of avian preen gland secretions is centuries old (see chapter 1, Elder 1954), yet, the controversy, or confusion, is still paramount. In fact, we have barely begun to formulate hypotheses and to test assumptions and predictions underlying seasonal and interspecific variation in preen wax composition. This thesis is a step in that direction. For red knots, and sandpipers in general, we now understand more about the selection pressures which play a role in the seasonal variation in preen wax composition, and which do not.

The research also makes clear that the question “What is the function of the preen gland of birds?” should include specifications about species, life history stage and sex. It is possible that age of individual birds also plays a role;

Kolatukuddy *et al.* (1991) showed that preen wax composition of mallard ducklings changes as soon as they replace the down with their first adult feathers. Most studies so far have not taken these aspects into account and function(s) of preen gland secretions were studied on different bird species, while chemical composition of the gland secretions was often unknown or not accounted for. Because of the great variation both within and between species, in the composition of preen gland secretions, it will be hard if not impossible, to find a general function of preen wax if such a general function would exist at all. For example, based on the results obtained on red knots of the study in chapter 5 one might conclude that preen wax has no function in plumage coloration of birds. However, it is known that greater hornbills *Buceros bucornis* dye their plumage with preen wax (Kemp 2001). Another example is the penetrating smell of preen gland secretions of hoopoes *Upupa epops* and red-billed woodhoopoes *Phoeniculus purpureus* that has an important function in repelling vertebrate nest predators (Law-Brown 2001, Martin-Platero *et al.* 2006), while in contrast to this, red knots might experience less nest predation by applying a less volatile preen wax mixture onto their feathers during incubation (chapter 7). It is the combination of chemistry, physics and evolutionary biology that will yield a greater understanding of causes and function of the large variation in avian preen wax chemistry. Below caveats in all relevant research fields are pinpointed and discussed and suggestions are done for an approach in which several research fields are integrated.

Understanding variation in preen waxes: a general explorative approach

Advanced organic chemistry is necessary to properly identify and describe the variation in chemical compounds secreted by preen glands. Complete descriptions of preen wax composition will help to generate valuable hypotheses that can be tested by evolutionary biologists. Chemical techniques have improved much over the last decades. While most chemical studies on preen wax have been performed on the hydrolysed products of the secretions, at present it is possible to study the intact components more easily (Dekker *et al.* 2000, Sinninghe Damsté *et al.* 2000, Burger *et al.* 2004), which increases our knowledge of preen wax compositions. Only few studies, have included volatile components of preen gland secretions (see Soini *et al.* submitted manuscript, for an exception), whereas such volatile components are known to be important in bird communication and chemical defense (e.g. Hagelin *et al.* 2003, Douglas *et al.* 2005a,b). Also, little is still known about the biochemical pathways of preen wax production (Jacob 1976).

Proper chemical analyses of preen wax composition of more bird species of

different ages, sexes and body conditions and in different habitats and seasons may result in more testable hypotheses about functional aspects of variation in preen wax within and between species. Recently, seasonal variation in preen wax composition, like those in sandpipers, have also been described in some passerines (Haribal *et al.* 2005), but specific hypotheses that could explain the seasonal variation were not provided.

Understanding possible functions of different chemical compounds starts with understanding the physical properties of the different compounds. A nice example of an experiment to the mechanical properties of waxes in an ecological context is given by Buchwald *et al.* (2006), who studied yield stress, yield strain, stiffness and resilience of wax of different bee (sub)species. Studies in which the functional (physical and/or biological) aspects of different avian preen wax compositions are tested are rare. Measurements of physical properties such as volatility, viscosity, melting points, light absorbance and reflectance, water repellence (cf. Patel *et al.* 2001 and Kulkarni & Sawant 2002) of different preen gland secretions, especially in interaction with the surfaces they are applied onto, would increase our understanding of the variation in preen wax compounds and generate biological hypotheses. The physical aspects of separate preen wax compounds are presumably dependent on other compounds in preen wax mixtures. Therefore, physical properties of both isolated compounds and of complete preen wax mixtures should be studied. Some of these physical aspects have been studied in the context of this thesis on mono- and diester preen wax mixtures of red knots, but this approach could be extended to many more preen wax mixtures of many species.

Preen waxes are products of living animals and variation in preen wax composition is the result of evolution by natural selection. The balance of costs and benefits of different preen wax mixtures - which is related to their chemical and physical aspects- determines the evolutionary process. Identification and measurements of these costs and benefits is a task for evolutionary biologists. Below three approaches for biological research are proposed that might bring us further in understanding variation in preen wax composition.

The fate of preen wax on the plumage: a descriptive approach

Better knowledge of the fate of preen waxes, once smeared onto the feathers, represent the kind of basic knowledge necessary to better understand preen waxes. Still a lot can be learnt about the fate of preen wax on feathers. For example, it is unknown whether preen wax is equally spread over all body parts and whether the wax forms a closed layer or small wax droplets on the feathers. Such

information is necessary to understand how waterproofing or anti-parasitic aspects of preen waxes would work, if such effects would exist at all. It has been suggested that preen wax penetrates into the shafts and barbs of feathers (Rutschke 1960), but verification of this suggestion is necessary. The relative, quantitative and qualitative, roles of integumental lipids and preen waxes deserves research attention (Stettenheim 2000) too, for example in the context of water repellence.

Investigating the turnover rate of preen waxes on the plumage is important as it will tell us whether changes in composition of preen wax produced by the preen gland is in correspondence with wax composition on the feathers. In a small study in which preen wax was simultaneously sampled from feathers and the preen gland, in captive red knots in spring, when they changed from mono- to diester preen waxes, confirmed a close correspondence between produced wax composition and composition of waxes on the feathers (M. Dekker unpubl. data). It is also unclear how stable preen waxes are, once smeared onto the plumage. This is likely dependent on the chemical composition of the waxes. Unsaturated waxes are likely less stable than saturated wax esters due to oxidation. Sweeney *et al.* (2004) found that although wax esters were still present in plumages of museum specimens of more than hundred years old, that these were mainly esters of saturated fatty acids and particularly lacked esters based on unsaturated fatty acids, presumably due to oxidation during the years of storage (Sweeney *et al.* 2004). Although these data show that preen waxes do not last on plumages forever, they are likely to stay on feathers for a longer period than the life span of most birds. Natural selection might act on the low stability of preen wax consisting of unsaturated fatty acids, and be the reason why preen waxes of most birds hardly contain unsaturated fatty acids. There are more chemical properties than saturation only, that determine the stability of preen wax (e.g. branchiness of the carbon chains) and properly designed experiments are needed to learn more about the stability of preen wax on feathers.

Feather mites, that probably do not occur on museum specimens, may consume preen wax (Blanco *et al.* 2001). Given the presumed stability of preen waxes, one might wonder what Blanco *et al.* (2001) meant when they stated that feather mites may be beneficial to birds as they may "remove old oil". Galván & Sanz (2006) showed that there is a positive correlation between feather mite load and the size of the preen gland of great tits *Parus major*, suggesting that plumicolous feather mites are ectosymbionts that use preen wax as their main food source.

More research is necessary on the interaction between preen wax and ectosymbionts. The huge variety in ectosymbionts may (partially) explain the variation in preen wax composition (Haribal *et al.* 2005). One might consider diet

studies on feather mites, using stable isotopes as markers and tracers of their food chain (e.g. Herman *et al.* 2000). Several experiments are possible using different feather mite species and different (labelled) preen waxes, that may resolve hypotheses concerning co-evolution between ectosymbionts and preen wax composition of the host species.

One aspect of preen waxes that should also be taken into account, and that has been ignored in this thesis, is quantitative variation in preen wax production. Bhattacharyya and Chowdhury (1995) showed that the preen glands of red-vented *Bulbuls Pycnonotus* cafer are substantially heavier and contain more lipids during the breeding season than in the non-breeding season. It is possible that the amount of preen wax applied onto feathers also changes seasonally in (some) birds.

The evolution of preen wax composition: a general, hypothesis-generating approach

In numerous studies, Jacob and co-workers have compared preen wax composition of many bird species for taxonomical purposes (e.g. Jacob & Poltz 1973, Jacob & Grimmer 1975, Jacob 1978, Jacob 1981 Jacob & Ziswiler 1982, Hoerschelmann & Jacob 1992, Jacob & Hoerschelmann 1993). In several of these studies, the relatedness of different bird species was based on the variation in species-specific preen wax composition, an approach which was called 'chemotaxonomy'. These chemical studies have provided detailed descriptions of species-specific preen wax compositions. However, they do not reveal much about the selective forces that have caused the inter- and intraspecific diversity in preen wax composition. It is also questionable how useful taxonomy is that is based on a single phenotypic trait that is likely subject of specific, directional selection pressures. Another problem with such 'chemotaxonomy' is that preen wax composition -at least in sandpipers- is an example of phenotypic flexibility (Piersma & Drent 2003) in which genotypes (individuals) express different phenotypes during an annual cycle (chapters 2, 3 and 4). If preen wax of different sandpiper species is sampled in different seasons, this will cause flaws in the taxonomy.

Biologically more interesting than the compilation of a 'chemotaxonomy' based on preen wax composition, is to search for ecological factors that may have lead to the diversity in preen wax composition. A general approach to try to pinpoint such selection processes is to map preen wax chemistry, or physical aspects of complete preen wax secretions, onto phylogenetic reconstructions and search for general patterns. For example, one might investigate whether birds in aquatic environments have distinctly different preen wax compositions than non-aquatic birds and whether the phylogenetic similarities are stronger than ecological simi-

larities. In box B, we attempted to use interspecific similarities between preen wax compositions to draw conclusions about the (time scales of) selection pressures that act on them. Although I think this is a useful method the analysis in box B, that contained only six species, was rather limited. The analysis, using many more different species, could indicate how evolutionary conservative certain preen wax compounds are, whether certain compounds occur more often in certain clades, or in different species among clades that share a certain ecological niche. It is intriguing, for example, that lapwings *Vanellus vanellus*, a species that shares habitats with black-tailed godwits *Limosa limosa*, at least during reproduction, show no seasonal variation in preen wax composition (Box C). One might infer from this that selection pressures causing changes in preen wax composition are not very strong (cf. Sweeney *et al.* 2004). The fact that male buff-breasted sandpipers *Tryngites subruficollis* and some individual male curlew sandpipers *Calidris ferruginea*, that do not incubate, still produce (small amounts of) diester preen waxes suggests that it is an evolutionary remnant from periods when males and females shared incubation in these species (Borowik & McLennan 1999) and that the costs of maintaining the biochemical and physiological machinery to produce diesters is not particularly high (chapter 3).

Costs and benefits of preen wax mixtures; experimental approaches

Although we think that diester preen waxes might be beneficial to diminish predation risk of offspring (chapter 7), it is still unclear why diesters are not secreted year-round. In other words, it still needs to be investigated which costs are involved in the use of diester preen waxes relative to monoesters waxes in non-breeding conditions. Experiments such as reported in chapter 5, 6 and 7, in which the functional aspects of different preen wax mixtures are investigated, may show that monoesters perform better than diesters. Thus far, no evidence exists for advantages of the use of mono- rather above diesters. It would be recommendable to involve both distinct monoester types ('A' and 'B', chapters 3 and 4) secreted during an annual cycle in such studies. Such an experimental approach is also useful in interspecific comparisons.

In addition to differences in functional aspects of different preen wax secretions, diesters also may be more energetically costly to produce than monoesters. As shown before there are no indications that there are substantial consequences for time budgets of birds in the application of preen wax, as it costs only little time to apply preen wax onto feathers. Ideally, it would be possible to manipulate preen wax composition in live birds and investigate energetical, functional and survival consequences.

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Nederlandse samenvatting

Waarom produceren steltlopers ander stuitklierwas tijdens het broedseizoen?

Tijdens de rui vervangen vogels hun versleten veren met verse exemplaren. Bij een versleten verenkleed nemen de wendbaarheid en de isolatie van de vogel sterk af. Het ruien zelf maakt een vogel echter ook kwetsbaar, omdat groeiende veren gemakkelijk breken en omdat vogels tijdens het aanleggen van nieuwe veren minder goed geïsoleerd zijn en ze voor de productie ervan extra moeten eten. Vogels kunnen veerslijtage dus beter beperken. Ze besteden dagelijks dan ook veel tijd aan het onderhoud van hun verenkleed. Door regelmatig een bad te nemen houden ze hun veren schoon. Met de snavel houden vogels de veren op hun plaats en worden luizen en mijten verwijderd. Ook het invetten van het verenkleed met was uit de stuitklier hoort bij de dagelijkse poetsactiviteiten.

De stuitklier bevindt zich aan de onderrug van een vogel, vlak boven de staart. In het onderhuidse deel van de klier wordt doorlopend een mengsel van vetachtige substanties geproduceerd, dat door een vlezig uitstulpinkje uitgescheiden wordt. Aan het uiteinde van de uitstulping zit een pluimpje korte, stugge veertjes. Het wasmengsel wordt met de snavel over het verenkleed gesmeerd. Tijdens de dagelijkse poetsactiviteiten raken vogels met razendsnelle bewegingen de stuitklier kort aan. Het subtiele masseren met de snavel stimuleert de klier om kleine hoeveelheden stuitklierwas uit te scheiden.

Stuitklierwas is vettig en dus waterafstotend. Het ligt daarmee voor de hand dat het droog houden van de veren en huid één van de functies van stuitklierwas is. Toch is het bewijs voor deze functie nog niet geleverd. Van Rhijn (1977) vergeleek hoeveel water veren van verschillende lichaamsplekken van een zilvermeeuw absorbeerden voor- en nadat hij de stuitklierwas chemisch verwijderde. Slechts in drie van de twintig gevallen bevatten de veren meer water na het verwijderen van de was. Wel namen juist de veren uit de omgeving van de stuitklier meer water op na ontvetting. De waterafstotende functie van stuitklierwas lijkt dus beperkt te zijn. Waarschijnlijk vormt stuitklierwas ook een beschermend laagje tegen overmatige veerslijtage en houdt het de veren soepel (Elder 1954). Een overzicht van de bestaande kennis met betrekking tot stuitklierwas is weergegeven in hoofdstuk 1 van dit proefschrift.

Seizoensgebonden veranderingen van stuitklierwas

De aanleiding van het in dit proefschrift beschreven onderzoek betreft de waarneming van Piersma *et al.* (1999) dat de chemische samenstelling van stuitklierwas van kanoeten in het voorjaar in korte tijd verandert. Kanoeten zijn middelgrote wadvogels die zo'n twee maanden per jaar (juni - juli) in hoogarctische gebieden leven voor de voortplanting. Om vanuit hun overwinteringsgebieden in West-Afrika of Noordwest-Europa hun Arctisch gelegen broedlocaties te bereiken moeten ze jaarlijks lange afstanden vliegen van vijf- tot zestienduizend kilometer. De verandering in de samenstelling van stuitklierwas vindt plaats wanneer de ka-

noeten op het punt staan naar de broedgebieden te trekken (hoofdstuk 2). In het laboratorium van het Koninklijk Nederlands Instituut voor Onderzoek der Zee (NIOZ) werd met gaschromatografie en massaspectrometrie de chemische samenstelling van de stuitklierwas gekarakteriseerd (Dekker *et al.* 2000, Sinninghe Damsté *et al.* 2000). Met deze methoden worden stoffen gescheiden op grond van hun vluchtigheid, waarna moleculen ervan in stukjes worden geschoten. Karakterisering geschiedt op grond van de molecuulspecifieke verdeling van de kapotgeschoten moleculaire brokstukken. Normaal gesproken scheiden kanoeten olieachtige mengsels van zogenoemde mono-esters uit. Die worden in het voorjaar vervangen door een kaarsvetachtig mengsel van di-esters (Piersma *et al.* 1999; fig. 2.1). In gevangenschap bleken kanoeten gedurende een jaar twee mengsels mono-esters uit te scheiden. Tussen half juli en eind maart wordt een mengsel van mono-esters uitgescheiden dat we 'mono-esters A' genoemd hebben. Vlak voor de vogels in juni en juli overschakelen op het gebruik van di-esterwas wordt nog een derde soort was uitgescheiden: mono-esters B. Dat scheiden de vogels ook gedurende ongeveer een week uit, vlak voordat de di-esters weer vervangen worden door de gebruikelijke was van mono-esters A (figuur 4.3). Door kanoeten meerdere jaren aan een constante daglengte van twaalf uur licht te onderwerpen en ze daarmee elke clou van seizoensverloop te ontnemen, werd ontdekt dat de samenstelling van de stuitklierwas nog steeds veranderde, maar dat de timing ervan afweek van kanoeten die de natuurlijke veranderingen in daglengte ervaarden. Het uitscheiden van di-esters viel altijd samen met de toename in lichaamsgewicht ter voorbereiding van de voorjaarstrek, ook als dit door het constante daglicht in bijvoorbeeld de herfst of winter plaatsvond. Hieruit blijkt dat veranderingen in stuitklierwas door een interne biologische klok aangestuurd worden en niet plotseling door vogels in gang kan worden gezet als dat nodig is (hoofdstuk 4).

De ontdekking van de seizoensgebonden veranderingen in stuitklierwas bij kanoeten roept de vraag op waarom strandlopers niet uit kunnen met een enkel stuitklierwasmengsel gedurende het hele jaar. De functionele aspecten van de verschillende wasmengsels staan centraal in dit proefschrift, waarbij mijn collega's en ik ons vooral richtten op de verandering van mono-esters naar di-esters.

De samenstelling van stuitklierwas

Voordat we ons richtten op de functies van de verschillende wasmengsels, was het handig een goede scheikundige beschrijving van de verschillende componenten in de wasmengsels te krijgen. De chemische samenstelling hangt immers samen met allerlei eigenschappen zoals smeltpunt, geur, kleur, viscositeit en daarmee de biologische functies. De samenstelling van de wasmengsels is voor iedere vogelsoort anders. Binnen een vogelsoort vertonen individuele vogels echter

weinig verschil in stuitklierwassamenstelling. Dat geldt ook voor kanoeten. Alle kanoeten vertonen dezelfde verandering van stuitklierwas en produceren gedurende een jaar mono-esters A, mono-esters B en di-esters (hoofdstuk 4). In het verleden heeft men op basis van de verschillende soortspecifieke wasmengsels evolutionaire stambomen opgesteld. Toen was echter nog niet bekend dat de samenstelling van de stuitklierwas kon veranderen. Het vergelijken van stuitklierwas van bijvoorbeeld een drieteenstrandloper in juni met dat van een steenloper in januari zal een heel andere stamboom opleveren dan wanneer de wassamenstelling van beide vogelsoorten in dezelfde tijd van het jaar worden vergeleken.

Om inzicht te krijgen in de evolutie van soortspecifieke wasmengsels, hebben we in detail de samenstelling bestudeerd van zes strandlopers. Dit waren twee pleviersoorten, twee strandlopersoorten en twee soorten grutto's. In elke familie betrof het een soort die buiten het broedseizoen uitsluitend in zoutwatergebieden verblijft en in het hoge noorden broedt, en één die 's winters in zoetwatergebieden leeft en wat zuidelijker broedt. De di-esters bleken te bestaan uit wasmoleculen met diolen (een alcohol met twee alcoholgroepen) of β-hydroxy vetzuren als basis. Diolen vormen verbonden met twee vetzuren een di-ester, β-hydroxy vetzuren in combinatie met een alcohol en een vetzuur (Box B, fig.1.2).

We onderzochten welke van de vogelsoorten de grootste overeenkomsten in de samenstelling van het di-esterwas vertonen. Zo hoopten we uitspraken te kunnen doen over wat het meest van invloed is op de samenstelling: de afkomst of het milieu (Box B). De samenstelling van de was bleek vooral te verschillen tussen de families waartoe vogelsoorten behoren, en veel minder tussen de milieus waarin die vogels leven. Of vogels overwinteren in bijvoorbeeld zoet- of zoutwatergebieden heeft weinig invloed op de wassamenstelling. Daarom verschillen die samenstellingen tussen de soorten waarschijnlijk al sinds miljoenen jaren (Box B). Een nog gedetailleerdere chemische analyse van de stuitklierwas is echter nodig om meer zekerheid te krijgen over hoe conservatief de soortspecifieke samenstelling van stuitklierwas is.

Make-up bij kanoeten?

Di-esters hebben een hoger smeltpunt dan mono-esters en zijn daardoor stijver en waarschijnlijk moeilijker op het verenkleed te smeren. Di-ester stuitklierwas werd bij een kanoet voor het eerst waargenomen tijdens de balts in de broedgebieden van Ellesmere Island, Noord-Canada (Piersma *et al.* 1999). In de broedgebieden lijkt zo'n stroperige di-esterwas lastig. De was laat zich in de lage temperaturen daar even stroef smeren als roomboter uit de koelkast. Er zou dus een voordeel aan de moeilijker smeerbare was verbonden moeten zijn dat opweegt tegen dit nadeel. Misschien functioneert een laagje di-esters op het verenkleed als een soort make-up die de kleurintensiteit of glans van het broedkleed ver-

sterkt? Als vogels tijdens de balts laten zien dat ze naast het veroveren en bewaken van een territorium extra tijd en moeite kunnen steken in het onderhoud van hun veren, zou dat voor een eventuele toekomstige partner een signaal kunnen zijn dat die vogels gezond en dus aantrekkelijk zijn (Delhey *et al.* manuscript). De mogelijke extra investering in het poetsen wordt dan terugverdiend met een aantrekkelijke partner en gezond nageslacht (Piersma *et al.* 1999).

Wij kunnen bij kanoeten met het blote oog geen kleurverschil zien tussen veren met mono-ester dan wel di-ester stuitklierwas. Misschien zijn dergelijke veranderingen heel subtiel en onzichtbaar voor het menselijk oog. Vogels kunnen ultraviolet licht waarnemen, dat voor mensen onzichtbaar is (Burkhardt 1989). Met een spectrofotometer hebben Peter Korsten en ik de kleurintensiteit van kanoeten in het voor vogels zichtbare lichtspectrum gemeten, toen ze mono-esterwas en, enkele weken later, di-esterwas produceerden. Er bleek geen meetbaar verschil te zijn tussen mono- en di-esters in de hoeveelheid gereflecteerd licht en de kleur van het verenkleed. Ook verwijdering van stuitklierwas van het verenkleed met een oplosmiddel beïnvloedde de kleur niet. Dit laatste verbaasde ons enigszins omdat een wattenstaafje zichtbaar geel kleurde als we een uitstrijkje van de stuitklier maakten. Bovendien hadden we ook de lichtabsorptie van puur stuitklierwas gemeten en daaruit bleek dat di-ester was meer, vooral ultraviolet licht absorbeert, vergeleken met mono-ester stuitklierwas. Waarschijnlijk is het laagje was op de veren te dun om een zichtbaar effect te bewerkstelligen. In ieder geval heeft een veranderde was-samenstelling zeer waarschijnlijk geen visuele betekenis voor kanoeten (hoofdstuk 5).

Alleen broedende steltlopers produceren di-ester stuitklierwas

Samen met Theunis Piersma en Jaap Sinninghe Damsté heb ik stuitklierwas van negentien strandlopersoorten, zes pleviersoorten en van de scholekster bestudeerd, dat door vele tientallen vrijwilligers met een wattenstaafje werd verzameld in verschillende perioden van het jaar. Na bestudering van de stuitklierwas bleek dat alle strandlopers en de meeste broedende plevieren de speciale di-esterwas produceren tijdens het broeden (fig. 2.2). Op het moment dat steltloperkuikens het nestkuiltje verlaten verandert de stuitklierwas van de ouders weer abrupt in de gebruikelijke mono-ester samenstelling (hoofdstuk 2). Dit patroon vonden we niet alleen bij steltlopers die op de toendra broeden, maar ook bij in Nederland broedende tureluurs, grutto's en scholeksters (hoofdstuk 2).

Een belangrijke ontdekking is dat steltlopers waarvan slechts één van de ouders voor het bebroeden van de eieren zorgt, de verandering van mono-ester naar di-ester secretie alleen bij de broedende vogel plaatsvindt (hoofdstuk 3). Bij kemp-hanen, blonde ruiters en krombekstrandlopers bebroedt alleen het vrouwtje de eieren en produceert ook alleen zij stuitklierwas die bestaat uit di-esters.

Daarentegen zijn het van rosse franjepoten juist de mannetjes die voor de broedzorg opdraaien en die di-esters uitscheiden (hoofdstuk 3). Dat soms maar in één van de seksen een verandering in stuitklierwas plaatsvindt is ook beschreven voor wilde eenden, waarbij de stuitklierwas van de vrouwtjes tijdens het broeden van mono-esters in di-esters verandert, terwijl de niet broedende woerden het hele jaar door onveranderd mono-esters produceren (Jacob *et al.* 1979; Kolattukudy *et al.* 1987).

Van vergelijkingen van stuitklierwas tussen vogelsoorten in verschillende perioden van het jaar hebben we dus geleerd dat de productie van di-esterwas voorkomt bij de meeste strandlopers en plevieren en bovendien bij de scholekster en de wilde eend. Dit zijn allemaal op de grond broedende soorten die tijdens het broeden di-esters op hun veren smeren. Deze constatering is een belangrijke stap in de richting van een functionele verklaring voor seizoensgebonden veranderingen in stuitklierwas.

De vergelijkende aanpak heeft ook geleid tot de ontdekking van enkele merkwaardige uitzonderingen. Zo blijken niet alle steltlopers di-esters te produceren in het broedseizoen. Kieviten, waarvan het verenkleed veel doffer wordt zodra ze eieren gelegd hebben -een waarneming waar de orginele suggestie voor de rol van di-esters nota bene op was gebaseerd (Piersma *et al.* 1999)- produceren het hele jaar door dezelfde stuitklierwas van mono-esters (Jukema *et al.* 2003). Ook morinelplevieren en strandplevieren schakelen niet over op di-esters tijdens de eileg en het broeden (Box C). De vraag waarom deze drie steltloopersoorten een uitzondering vormen kan pas beantwoord worden als we meer inzicht hebben in de functie van seizoensgebonden veranderingen in de samenstelling van stuitklierwas bij andere soorten.

Verbeterde bescherming tegen veerslijtage?

Naast het waarschijnlijk waterafstotende effect van stuitklierwas is ook geopperd dat de was slijtage van veren voorkomt (Jacob & Ziswiler 1982). Het zou kunnen dat de tijdens het broeden uitgescheiden di-esters de veren beter beschermen dan mono-esters. Dat zou nuttig kunnen zijn omdat de veren van de broedende vogels veel in contact komen met de grond. Hier probeerde ik samen met enkele collega's grip op te krijgen met een experiment met verschillende soorten broedende strandlopers in Noordoost-Groenland. We vingen bonte strandlopers, drieteenstrandlopers, steenlopers en kanoeten op het nest. Nadat we van één van de vleugels de was hadden verwijderd met oplosmiddel, lieten we de vogels weer los, om ze enkele dagen later opnieuw te vangen. Door de vleugelpunten voor en na deze behandeling nauwkeurig te bestuderen door een microscoop en de slijtage te beoordelen, probeerden we te achterhalen of de vleugelpunt zonder was sneller sleet dan de onbehandelde vleugelpunt. Dit bleek niet het geval. Beide

vleugels waren nauwelijks extra versleten bij de tweede controle, dus het is mogelijk dat het experiment te kort duurde om (een verschil in) veerslijtage te constateren. Natuurlijk kunnen we niet uitsluiten dat de vogels vrijwel direct nadat wij de stuitklierwas van de vleugel verwijderden deze weer voorzagen van een vers waslaagje, maar het spul was op zijn minst korte tijd afwezig (Box D).

Bestrijdingsmiddel tegen bacteriën?

Vogels herbergen verschillende bacteriën in hun verenkleed die de veren langzaam afbreken (Burtt & Ichida 1999). Om te voorkomen dat deze parasieten het verenkleed beschadigen moeten vogels zich hiertegen wapenen. Bacteriën groeien meestal goed in warme, vochtige omstandigheden. Tijdens het broeden creëert een vogel een microklimaat dat niet alleen gunstig is voor de eieren, maar ook voor veerafbrekende bacteriën. Juist tijdens het broeden zou extra bescherming daartegen handig zijn. De effecten van zowel mono-ester als di-esterwas op de groei van de veerafbrekende bacterie *Bacillus licheniformis* werd bestudeerd door de borstveren van kanoeten in een vloeistofmengsel met voeding met bacteriën te plaatsen. Dit werd gedaan met veren waar de vogels zelf mono- of di-esterwas opgesmeerd hadden en met veren waarvan beide wasmengsels waren verwijderd. Als afbraakproduct van keratine, de bouwstof van veermateriaal, ontstaan kleine deeltjes (zogenoemde oligopeptiden) die licht absorberen dat door de bacteriekweek geschenen wordt. Lichtabsorbtie is daarmee dus een mooie maat voor bacteriële veerafbraak. Beide wasmengsels bleken de groei van deze bacteriën te remmen, maar mono- en di-esters waren daarin even effectief. We denken dat stuitklierwas de bacteriën niet chemisch bestrijdt, maar dat een laagje was op de veren als een fysieke barrière tussen veer en bacterie de veerafbraak belemmert (hoofdstuk 6).

Geur-camouflage?

Steltlopers broeden op de grond en zijn dus kwetsbaar voor roofdieren die het op hun legsel gemunt hebben. De enige manier om predatie te voorkomen is door zo onopvallend mogelijk de eieren te bebroeden. Steltlopers hebben daarom een uitstekend camouflerend verenkleed. Veel roofdieren, zoals vossen en hermelijnen, gebruiken hun neus bij het opsporen van prooi. Grondbroeders doen er dus goed aan zo weinig mogelijk geur te verspreiden. Di-ester stuitklierwas smelt bij een hogere temperatuur dan mono-ester was. Di-ester was is dus minder vluchtig en daardoor waarschijnlijk moeilijker te ruiken voor roofdieren. Dit hebben we getest met een herdershond die Ton van der Heide trainde om mono- en di-ester stuitklierwas op te speuren. De hond moest in een experimentele opstelling van zes buizen ruiken in welke buis stuitklierwas van kanoeten aanwezig was. Ton en ik bekeken hoe vaak de speurhond het buisje met stuitklierwas succesvol lokali-

Een goed getrainde speurhond werd ingezet om te achterhalen of mono-ester was van kanoeten beter te ruiken is dan di-ester was.

seerde. De hoeveelheid was van beide mengsels varieerde van 0,24 tot 16 microgram. Na twintig proeven met elke mogelijke combinatie van hoeveelheid en samenstelling van de was bleek dat de speurhond meer moeite had de kleinere hoeveelheden stuitklierwas op te sporen en dat zij bij lagere hoeveelheden vooral di-esters moeilijk wist te vinden (hoofdstuk 7). Het uitscheiden van di-ester was levert steltlopers waarschijnlijk dus een voordeel op tijdens het broeden, doordat het de kans op predatie van het nageslacht vermindert.

Kosten en baten van veranderingen in poetswas

Als di-ester was zo'n voordeel heeft, waarom zouden steltlopers het dan niet ook buiten het broedseizoen op hun veren smeren? Waarschijnlijk is de productie of het gebruik van di-esters kostbaar. Een geur-camouflerende werking vergroot weliswaar de kans op nageslacht, waarschijnlijk staan er kosten of nadelen tegenover. Maar welke nadelen zijn dat?

Zoals eerder beschreven kost het misschien meer tijd om de moeilijker smeerbare di-esters op het verenkleed aan te brengen, vergeleken met de tijd die een vogel kwijt is aan het smeren van mono-esters. Uit zes uur observatie van het poetsgedrag van zes kanoeten in gevangenschap, bleek dat de vogels maar heel weinig tijd besteden aan het invetten van de veren. Hoewel veeronderhoud dagelijks best wel wat tijd in beslag nam: gemiddeld 4 minuten en 20 seconden per uur (7.2 % van de tijd) en de vogels zo'n vier keer per uur hun verenkleed invetten, kon met gedetailleerde video-analyse bepaald worden dat het smeren van was op de veren slechts 54 seconden per uur kostte (Box A). Bij slechts 2,9 procent van alle dagelijkse poetsbeurten vond contact met de stuitklier plaats. Dat getal komt goed overeen met de 3,1 procent bij boerenzwaluwen (Møller 1991). Het is moeilijk voor te stellen dat een activiteit die dagelijks zo weinig tijd in beslag neemt veel energie kost, zelfs als het smeren van di-esters twee keer zo veel tijd zou kosten als het smeren van mono-esters. Waarschijnlijk spelen andere kosten een grotere rol.

Het is mogelijk dat voor de productie van di-ester stuitklierwas bepaalde enzymen of hormonen nodig zijn waarvan hoge concentraties in het bloed energie kosten of negatieve bijwerkingen hebben. Dan zou niet het smeren van di-esters meer energie kosten, maar de productie ervan. Omdat de productie van stuitklierwas nog nauwelijks onderzocht is, blijft de vraag naar het energieverbruik voor de productie van mono- en di-esters vooralsnog onbeantwoord.

Om de evolutie van verschillen in stuitklierwassamenstelling beter te begrijpen is van veel verschillende vogelsoorten in verschillende omgevingen en seizoenen een goede scheikundige beschrijving nodig van de wassamenstelling. Door die te relateren aan relevante fysische eigenschappen, zoals waterafstotendheid, kleur en geur, kunnen ideeën rijzen over de kosten en baten van verschillende soorten stuitklierwas. Die ideeën kunnen vervolgens experimenteel onderzocht worden. Veel onduidelijkheid bestaat er bijvoorbeeld nog over hoe lang stuitklierwas op het verenkleed aanwezig blijft en wat kanoeten er telkens toe beweegt de was op hun veren te vervangen. Het lijkt er steeds meer op dat de in dit proefschrift beschreven veranderingen in en functies van stuitklierwas bij vogels nog maar een topje van de ijsberg vormen. Waarschijnlijk liggen er nog veel spectaculaire ontdekkingen in het verschiet (hoofdstuk 8).

Voor het doen van zulke ontdekkingen is het aan te raden organische chemie en experimenteel biologisch onderzoek te combineren. Het is deze unieke combinatie van een goed organisch chemisch laboratorium en de wereldwijd unieke faciliteiten voor experimenteel wadvogelonderzoek, vergezeld van kundige experts in beide vakgebieden, die samengebracht op een enkel instituut op Texel, het NIOZ, hebben geleid tot de wetenschappelijke ontdekkingen die beschreven zijn in dit proefschrift.

Dankwoord /Acknowledgements

Op 7 juli 2006, toen ik bezig was met de laatste loodjes van mijn proefschrift ontving ik, onafhankelijk van elkaar, en zonder dat ik om deze wijze raad gevraagd had, twee e-mails met daarin de volgende passages betreffende promotie-onderzoek:

> *"…. jarenlange noeste intellectuele arbeid en gedoe met speurhonden en wassen neuzen, … dat moet je ook alleen uit innerlijke drang doen en als iedereen vervolgens staat te juichen, is dat een meevaller."*

> *"Thank goodness my dissertation defense is behind me! It is a gear shifting experience. Here I have been going full speed for 6 years trying to do all this stuff…..The PhD is a big investment of time, money and effort."*

Ik kan bovenstaande uitspraken grotendeels begrijpen maar ik vind ze niet zo zeer van toepassing op mijn promotie-onderzoek. Mijn promotietijd heb ik voornamelijk als een erg mooie, leerzame periode ervaren. Het had met name met het gezelschap waarin ik me begaf te maken, dat de bovenstaande beschreven jarenlange noeste intelectuele arbeid tot een plezier maakte. Op deze plek wil ik de verschillende medeverantwoordelijken voor dit proefschrift dan ook graag even noemen en bedanken. Ik doe dat hier in semi-willekeurige volgorde. Bovendien hoop ik in dit hoofdstuk mijn ouders en 'schoonouders', die wel eens hun verbazing uitten over het feit dat ik vaak in het meervoud over mijn promotie-onderzoek sprak, duidelijk te maken dat wetenschap samenwerking is en dat het boekwerkje dat jullie nu in handen hebben alleen gemaakt kon worden met de hulp en morele steun van vele anderen.

Theunis, mijn dank voor jou is zonder twijfel het grootst van allen die ik hierna nog ga noemen in dit hoofdstuk. Al is het maar omdat het geheel aan jou ligt dat ik met velen die jou volgen in dit dankwoord in contact ben gekomen en dat dit allen erg plezierige mensen in de omgang bleken te zijn. Allereerst natuurlijk bedankt, Theunis, voor je onbegrensde vertrouwen in me en dat je mij uitkoos om

dit promotie-onderzoek uit te voeren. Ik had me werkelijk geen beter begeleider kunnen wensen; je had altijd zinnige antwoorden op mijn vragen, van welke aard ook en op welk tijdstip ook. Je voorzag manuscripten *altijd* zeer tijdig van commentaar, ook nadat je werkzaamheden zo'n beetje verviervoudigden met je hoogleraarschap, en ook als je je op moeilijk bereikbare plekken in het buitenland bevond. Daarnaast heb je vele, onvergetelijke en/of bijzonder nuttige mogelijkheden voor me gecreëerd die lang niet altijd van direct belang voor mijn promotie waren maar ongetwijfeld hun nut zullen bewijzen tijdens mijn verdere carrière. En anders waren het gewoon unieke ervaringen die ik niet had willen missen. Daarnaast waardeer ik ook je vriendschap, en de tijden die we samen over wat dan ook kletsend (maar meestal betrekking hebbend op strandlopers, wetenschap, de Waddenzee en natuurbescherming) in de NIOZ-kantine, in Gaast, op de Groenlandse toendra, het wad of de sepkha of, de laatste jaren, op het Marsdiep doorbrachten. Onze gezamenlijke expedities naar Groenland en Mauritanië, en de vele vangnachten op het wad zijn onvergetelijk en waren en zijn voor mij van zeer grote waarde. Ik kan dan ook alleen maar bijzonder gelukkig zijn met het feit dat ik nog drie jaar bij jou als postdoc aan de slag mag, ditmaal in Groningen werkend aan Drieteenstrandlopers. Ik ben ervan overtuigd dat dit vergelijkbaar mooie jaren worden als de voorgaande.

Wat voor Theunis geldt, geldt uiteraard ook voor mijn andere promotor. Jaap, bedankt dat je met 'de vogelaars', die nauwelijks benul van organische chemie hadden (hebben?) in zee wilde om aan dit toch wat vreemde project te werken en samen te publiceren in tijdschriften die je niet kende en alleen maar jouw gemiddelde *citation index* omlaag haalden (maar die voor een bioloog lang zo slecht niet zijn). Je had altijd een frisse, kritische blik op het onderzoek en jouw inbreng kwam onze artikelen en daarmee dit proefschrift altijd ten goede. Zonder de uitzonderlijke combinatie van jouw excellente lab en de wereldwijd unieke wadvogel-unit op het NIOZ had dit onderzoek nooit plaats kunnen vinden.

De goede wetenschappelijke en vooral ook uiterst gezellige sfeer op het NIOZ, met name binnen de 'vogelvleugel' is van onschatbare waarde geweest. Anne (2x), Anneke, Anita, Bernard, Casper, Danny, Debbie, Dennis, François, Fred, Gerhard, Henrike, Isabel, Jaap, Jan (x2), Jutta, Kees, Luisa, Maarten, Petra, Phil, Pim, Piet, Pieter, Pieternella, Sue, Tanya, Theunis en Wouter en alle anderen die voor langere of kortere tijd een belangrijke rol in mijn leven op en nabij het NIOZ hebben gespeeld: bedankt voor de vele goede en leuke gesprekken, literatuurbesprekingen, lol, samenwerking, steun en gezamelijke vogelwaarnemingen! Door de intensieve samenwerking en hechte band, ook buiten het NIOZ, zijn julie een soort tweede familie voor me geworden. Piet en Wouter wil ik speciaal bedanken voor het doornemen van enkele hoofdstukken in dit proefschrift en Jan Drent en Pieternella voor discussies over mijn werk. Een speciaal woord van dank

wil ik ook nog wijden aan de drie onmisbare krachten Anne, Bernard en Maarten die er voor zorgen dat alle onderzoek door vele promovendi, post-docs en studenten zo vlekkeloos verloopt. Jullie werk wordt, zeer ten onrechte, vaak als vanzelfsprekend ervaren. Ik hoop van harte ook in de toekomst nog met de NIOZ-vogelaars (en daar reken ik de benthos-lieden ook toe) samen te mogen werken of gewoon voor het plezier samen op pad te gaan. Met mijn toekomstige aanstelling in Groningen aan drietenen zal dat zeker gaan lukken: ik kijk er al naar uit!

Zonder dat velen het zich wellicht zullen realiseren, was Joop Jukema ook een belangrijke aanstichter van mijn promotieonderzoek. Zoals Joop wel vaker zijn ongelooflijk klinkende praktijkervaringen met betrekking tot goudplevieren, kemphanen en (rosse) grutto's samen met een heel team van wetenschappers verifiëert (en eigenlijk zelden falsifieert), zo vormde hij ook een belangrijke aanleiding voor mijn promotie-onderzoek. Als Joop achter zijn *skule* op Texel zijn verhaal over vaal kleurende kievitvrouwtjes niet met Theunis had gedeeld, dan had Theunis wellicht nooit besloten samen met Jaap een beurs aan te vragen voor dit onderzoek. Dat het 'mysterie van de *faele jok*' voorlopig een mysterie blijft en dat er slechts een box, en geen hoofdstuk, in dit proefschrift aan is gewijd betekent natuurlijk niet dat de er uit voortkomende hypothese over kanoeten make-up onbelangrijk is geweest. Integendeel. Joop, je liet er ook bepaald geen gras over groeien toen ik vroeg om stuitklierwas van kemphanen en kieviten. *Tige tank* ook Joop, voor je regelmatige, immer stimulerende en betrokken, telefoontjes. En wat een geweldig team waren we in Mongolië samen met Jan, Tseelee, Gantulga, Enghee en Bathaa. Ook aan die expeditie lag jij voor een belangrijk deel ten grondslag. Ik kijk al uit naar de gezamelijke stukken die we hier over gaan schrijven en ons bezoek aan het museum van Moskou.

Jan Wijmenga was ook onmisbaar voor het slagen van de expeditie naar Mongolië (hoewel het jammer blijft dat we niet de moeite hebben genomen de bijzondere kenmerken van Mongoolse piepers; tanden, beharing en het bewonen van holletjes, niet verder wetenschappelijk te beschrijven). We mogen in onze handjes knijpen dat Theunis ons met maar weinig tegenstrubbelingen naar de Mongoolse steppen liet vertrekken terwijl zowel jij, Joop als ik eigenlijk met steltloperonderzoek in Nederland bezig zouden gaan. Hoewel deze expeditie weinig van doen had met het onderwerp van dit proefschrift was het een zeer welkome afwisseling op mijn werk. Jan, ik hoop dat er snel ook een mooie aio-plek voor jou in het verschiet ligt.

Mijn enthousiasme voor en interesse in vogeltrek werd aangewakkerd door de tellingen van ongestuwde vogeltrek nabij Maria Hoop. Deze werden in belangijke mate geïnitieerd door Jan Boeren en Ran Schols, en later, en nu nog steeds intensief, door verschillende enthousiastelingen van de vogelwerkgroep de Haeselaar uitgevoerd.

In Churchill, Canada, is mijn enthousiasme voor steltloperonderzoek in hun broedgebieden ontstaan. Onder haar bezielende begeleiding deed ik samen met Karen Krijgsveld voor het eerst veldonderzoek aan steltlopers in (sub)arctische streken. De vele (nachtelijke) uren in een klein schuiltentje, luisterend naar pliepjes van zenders op de ruggetjes van Amerikaanse goudplevierkuikens met het gezoem van honderden, zo niet duizenden, muggen op de achtergrond zal ik niet licht vergeten.

Later on Guy Morrison allowed me to spend 3½ months in Alert. In many ways this was an unforgettable experience that resulted in several publications. I hope that a few more will follow. Clare Morrison contributed much to the fun in Alert.

I have learnt a great lot during the six months total that I spent in the lab of John Wingfield, under supervision of Marilyn Ramenofsky. I enjoyed these periods a lot. As opposed to my predictions and wishes then, I never got to do a PhD on hormones, but on 'those funny waxes' instead. Indirectly, however, the lab skills and theory that I was taught in Seattle have been useful during my PhD too. John, I particularly like to thank you for allowing me to join an expedition to Thule, Greenland together with Michael, Meta, Simone and Brian. It was a fantastic and enjoyable time during which I also collected some more preen waxes of incubating arctic shorebirds (and snow buntings and Lapland buntings), that have been used in chapter 2. Another comrade from my 'hormonal period' is Meta Landys. Meta, I enjoyed the many conversations (I shouldn't call them 'discussions', right?) and small and big bird catching trips that we had in the Netherlands, Greenland and the USA.

I like to acknowledge everyone that spent time with us in Zackenberg Research Station in Greenland. The enthusiasm of Hans Meltofte and the great facilities offered by the Danish Polar Centre made our trip in 2003 to a big success, a memorable time and was the occasion to my future post-doc on sanderlings. I much look forward to the coming three field seasons in Zackenberg. Wat geweldig dat ik dat eerste veldseizoen zeer waarschijnlijk met Joop, Koos en Hans en Ingrid mag doorbrengen! Hans en Ingrid wil ik verder bedanken voor gezellige vogel-tripjes, goede discussies en veel leuke gesprekken!

With all of my co-authors on chapter 3 I often had lively and fruitful scientific discussions about the evolution of changes in preen wax composition in sandpipers and other issues relating to the breeding biology of sandpipers. I also had many of such, and even more conversations during the annual International Wader Study Group Meetings. The network of friendly and enthusiastic shorebird-fanatics of the Wader Study Group enabled me to receive preen wax samples from several countries of each continent on this planet. Antarctica is the only exception, but I am sure I would have received those too if shorebirds had been more com-

mon there. I like to thank this small army that collected preen waxes on worldwide locations. Most of them are mentioned in the acknowledgements of chapter 2, but many others that added samples to my preen wax sample collection after the completion of chapter 2 are not. As I am sure that it is impossible to remember, or even know, everyone who was involved in these preen wax collections, I leave it to this general acknowledgement: thanks a great lot, without your contribution this thesis would have been far from what it is now! I want to apoligise to those whose samples are still laying untouched in the NIOZ-freezer. During the last six years I have analysed at least 6,335 preen wax samples (the gas chromatograph had to run at least for 158 days, 22 hours and 30 minutes to complete this job) and there are still 1,074 (14.5 %) samples that I never looked at. Dat de gaschromatograaf deze vele uren, vrijwel altijd netjes zijn werk deed is vooral te danken aan Michiel Kienhuis en Marianne Baas. Veel dank hiervoor!

Thanks also to Thomas Alerstam and Susanne Åkesson, for organising a very useful course on animal migration in Lund, Sweden. It is not a coincidence that many of the participants still encounter each other regularly during international congresses. One of these participants is Tómas Grétar Gunnarson. Tómas, I look forward to visit you in Iceland the coming years to work together on sanderlings.

Met Peter Korsten was het plezierig samenwerken aan hoofdstuk 5. Peter, ik waardeerde je gezelschap en inzet tijdens de weekenden van nota bene je eigen veldseizoen. Je nauwkeurigheid waarbij niks aan het toeval werd overgelaten heeft tot een betrouware analyse en gedegen publicatie geleid.

I am still glad that I met Jed Burtt at the International Ornithological Congress in Beijng in 2002 (and later again at the IOC in Hamburg) and that he was willing to cooperate on a project that evolved to chapter 6. That Amy visited NIOZ and the Dutch mudflats was also thanks to Jed. Amy, thanks a lot for your careful labwork that saved me a lot of time. Maaike Versteegh droeg eerst als cursist Dierecologie, daarna als zelfstandig doctoraalstudent samen met Jed, een grote verantwoordelijkheid voor het slagen van het experiment over bacteriële afbraak van veren en het gepubliceerd krijgen van dit onderzoek (hoofdstuk 6). Maaike, bedankt voor je inzet en gezelligheid!

Ton en Ella van der Heide waren eerst in Egmond-Binnen en later in Donkerbroek altijd gastvrij. Ton, ik waardeer je inzet voor het experiment met Joey, dat tot een van de acht hoofdstukken van dit proefschrift –en zeker niet het minst belangrijke– heeft geleid, ontzettend. Dat ik, afhankelijk van openbaar vervoer, wel eens te laat kwam en je daardoor geregeld liet wachten bij het station heeft jouw humeur volgens mij nooit beïnvloed; het was in ieder geval niet zichtbaar. Naast je inzet om Joey te trainen voor het experiment, ook al zaten de door jou verwachte beloningen er niet in, waardeerde ik onze gesprekken en de mogelijkheid om te overnachten tijdens de vele train-weekenden ontzettend.

I am grateful to David Winkler, Jan de Leeuw and Jan Komdeur for taking part in the reading committee. Thanks for accepting this task, I look forward to discuss my thesis with you during my defense.

With Barbara Helm and Kaspar Delhey I already have had some discussions concerning preen wax. Thanks for this, I enjoyed every e-mail I received of you and I hope they have prepared me for my final defense.

Oscar, aan jou lag het niet dat ik wel eens klaagde over Den Helder. Hoewel ik aanvankelijk moest wennen aan het wonen in een huis waar eerst veel tijd en moeite gestoken werd in een muurschildering nog vóór dat we een vloer in de woonkamer en fatsoenlijke douche hadden, mocht het uiteindelijke resultaat er zeker zijn. Volgens mij ben je mijn enige huisgenoot geweest die echt nooit gezeurd heeft over mijn slordigheid, al heeft dat waarschijnlijk alles te maken met het feit dat we niet voor elkaar onder hoeven doen wat die eigenschap betreft. Misschien moeten we de uitvinder van de afwasmachine gezamenlijk nomineren voor de Nobelprijs voor de Huisvrede. Het was erg gezellig met je aan de Piet Heinstraat, samen met juffrouw Janny, Bert, de gluurbuur, Sticky, Jip, Janneke en al die andere rare Heldenaren. Best jammer dat aan die tijd een einde is gekomen.

Ook de bemanning van de Navicula, Kees, Johan, Hein en Tony; van harte bedankt voor de gezelligheid en onmisbare hulp tijdens wadexpedities. De waarde van een eigen onderzoeksschip met een uitstekende bemanning als die op de Navicula mag niet onderschat worden. Als ik het zonder had moeten stellen, had ik geen experimenten met kanoeten in gevangenschap kunnen doen en het met veel minder stuitklierwasmonsters moeten stellen. En dan was ik nog een NIOZ-onderzoeker met relatief weinig onderzoeksbinding met het wad!

“Onderzoeksbinding”, want anderszins heb ik wel degelijk een sterke band met de Waddenzee. Binnen de groep wadecologen op het NIOZ werd er vaak gekscherend gedaan over mijn onderzoeksonderwerp; mijn collega-ecologen noemden het onderwerp van mijn onderzoek vaak als voorbeeld voor het soort onderzoek dat we als groep Mariene Ecologie en Evolutie vooral niet moesten nastreven. Misschien dat juist deze positie tussen echte wadecologen me ertoe bracht me anderszins bezig te houden met de Waddenzee. Iedereen die me heeft meegemaakt tijdens de afgelopen jaren weet dat ik niet altijd, zoals het een ijverig promovendus betaamt, alleen met mijn onderzoek bezig ben geweest. In de eerste maand van mijn aanstelling, en al even daarvoor, bereidde ik samen met Martijn de Jong en Petra de Goeij (die toen in Seattle was) en met goede steun van vele betrokkenen, met name Anne, Anita en Pieternella, een actie tegen schelpdiervisserij op het wad voor. Dit bleek later een opmaat voor de oprichting van Wilde Kokkels en een bijzonder interessante periode met spannende rechtzaken, nog meer acties, heel veel ‘gedoe in de media’, steunbetuigingen en bedrei-

gingen, gesprekken met Kamerleden en staatssecretarissen in Den Haag, verschrikkelijk veel e-mails aan en van mensen van allerlei pluimage, lezingen en veel (te veel) overleggen, inspraakbijeenkomsten en vergaderingen en uiteindelijk een verbod op mechanische kokkelvisserij in de Nederlandse Waddenzee. Toevallig of niet, handig of niet: deze periode viel vrijwel geheel samen met de periode waarin ik aan mijn promotie-onderzoek werkte. Wellicht was dit proefschrift één of twee hoofdstukken dikker geweest, of een jaar eerder af geweest, als ik me niet met het 'stelletje procedurevandalen' van Wilde Kokkels had ingelaten. Het zij zo. Lenze, Petra en Martijn: wat hebben we een hoop meegemaakt en bereikt met zijn vieren! Ons Wilde Kokkel-tijdperkje zal ik nooit vergeten. Ik ben blij dat Lenze en Martijn, de twee Wilde Kokkels die me de afgelopen jaren nog het meest van mijn wetenschappelijke werk hebben gehouden, me tijdens mijn verdediging willen bijstaan als paranimf. Ik wil hen en Petra ook bedanken voor hun begrip als ik af en toe, vooral in de afgelopen twee jaren, eens niet in staat was om iets voor Wilde Kokkels (tegenwoordig Stichting WAD) te betekenen. Iemand anders die me geregeld van mijn werk hield was Sandra Kloff. Sandra, wat zou het mooi zijn als alle natuurbeschermers dezelfde drive en doelgerichtheid hadden als jij!

Theunis, ik ben jou ontzettend dankbaar voor je onvoorwaardelijke steun, betrokkenheid en vrijheid die je me hebt gegeven voor mijn Wilde Kokkel-activiteiten en om mezelf ook op vele andere vlakken te ontwikkelen. We zullen nooit weten hoe belangrijk de rol van Wilde Kokkels is geweest in het uiteindelijke verbod van de mechanische kokkelvisserij. Ook zullen we nooit weten hoe mijn proefschrift er uit had gezien als LNV nooit vergunningen voor mechanische kokkelvisserij had verleend. Ik weet echter dat geen van ons tweëen spijt heeft dat het is gegaan zoals het is gegaan.

Ik wil Jan de Leeuw, toenmalig directeur van het NIOZ, van harte bedanken voor zijn steun door twee competente advocaten in te huren die 'Jeroen Reneerkens en het Koninklijk NIOZ' vertegenwoordigden nadat ik enkele dagen voor de oprichting van Wilde Kokkels, in mijn eentje, maar uiteraard bijgestaan door Lenze, Martijn en Petra, de onderzoeksbelangen van het NIOZ verdedigde bij de Raad van State tegen de schadelijke gevolgen van de mechanische kokkelvisserij. Piet, voor jou was het misschien een kleinigheid die je alweer vergeten bent, maar ik wil je ook nog ontzettend bedanken voor het helpen schrijven van die eerste pleitnota, tot diep in de nacht!

Ook tijdens de telweekenden op Vlieland met *Team Asadi* was de Waddenzee en alles wat er maar enigszins mee te maken had -of juist helemaal niet- een belangrijk gespreksonderwerp. Hoewel ik o.a. vanwege gebrek aan Asadi's nog niet volledig opgenomen ben in het team voel ik me er al sinds mijn eerste telling in thuis en bij betrokken. Peter, Romke, Lenze, Guus, Carl, Menno en Leon (en Kees

vast ook nog wel eens keer), bedankt voor de gezelligheid, de mooie waarnemingen, het bier, de whiskey, 's werelds beste kaasfondue en alle anders.

Naast de wadvogeltellingen heb ik me ook als redacteur voor Limosa bezig mogen houden met de ornithologie in Nederland. Alle redacteuren bedank ik voor een erg fijne tijd en samenwerking aan een prachtig tijdschrift de afgelopen jaren.

Dick Visser verzorgde op bijzonder prettige wijze de prachtige lay-out van dit proefschrift. Jan van de Kam voorzag me, zoals altijd geheel belangeloos, van enkele van zijn prachtige foto's die dit proefschrift verfraaien. Koos Dijksterhuis toonde zich een geïnteresseerde persmuskiet die twee zeer leesbare artikelen voor NRC Handelsblad over mijn (promotie-) onderzoek schreef. Daarnaast wil ik je bedanken, Koos, voor onze leuke e-mailwisselingen, voor het in de Dijkzathe op Schier kunnen uitrusten van aio-beslommeringen, voor je tip veel aandacht te besteden aan dit dankwoord, en natuurlijk voor het kritisch doornemen van de Nederlandse samenvatting. Ik hoop van harte dat onze gezamelijke plannen met betrekking tot drietenenonderzoek de komende drie jaar zo zullen uitpakken als we beiden in ons hoofd hebben. We zijn nog niet van elkaar af en daar ben ik bepaald niet rouwig om.

Al mijn Bloemsingel- (tegenwoordig Prinsenhof)vrienden, Arjèn, Thor, Dries, Martijn, Lenze, Jan en Azemith, Jochum, Angela en Jan dank ik voor voor gezamelijke vakanties, scoortripjes, nachtelijke kroegbezoeken, feesten, gezelschap en vriendschap. Lenze, het opperhoofd van dit bonte gezelschap, dank ik voor alle mogelijkheden die hij ons heeft geboden. Niet onbelangrijk daarbij waren de gratis concertkaartjes, vele rondjes in de kroeg en het altijd bieden van slaapplekken en zelfs een eigen kamer. Wat dat betreft hoef ik me bepaald geen zorgen te maken voor de komende jaren, nu ik weer in een prachtig Carex-pand mag komen wonen met jullie. Vooral Arjèn, was en is behalve een goede vriend ook altijd beschikbaar voor wat voor soort steun dan ook. Ik zal je de komende jaren zeker geregeld komen opzoeken in Liverpool voor gesprekken over vogels, muziek, fotografie, Pixies, Rembo & Rembo, Kamagurka en nog veel meer. Ik beloof dat ik tijdens jouw verblijf in Liverpool met Thor en Dries de 'scory soorten' in de Keeg in de gaten zal houden.

Mijn familie, en vooral mijn ouders, wil ik bedanken voor hun onvoorwaardelijke steun en vertrouwen in me. Zonder jullie, Jef en Nicole, had ik nooit zoveel interesse gehad in de wereld om me heen. Nicole, ik waardeer de bijzondere moeder-zoon relatie die we dankzij onze gezamelijke interesse in vogels, en de laatste jaren ook in natuurbescherming, hebben. Dit proefschrift is ook een beetje van jullie, al is het maar omdat het voor een beperkt deel de oorzaak is waarom ik jullie, tegen jullie wens in, niet vaker bezocht in Echt de afgelopen jaren.

Welmoed naast de mooie tekeningen, je hulp en gezelschap tijdens veldwerk in Groenland en op Griend (wat geweldig dat we samen op zulke mooie plekken mochten zijn!), het bieden van een fijne werkplek in Amsterdam en vele andere bijdragen aan dit proefschrift ben ik vooral heel erg blij dat ik mijn periode als promovendus samen met jou mocht doorbrengen. Ik hoop dat dat nog heel lang mag duren, maar dan niet meer als promovendus.

Amsterdam, 26 november 2006

List of publications

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